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Development, Optimization, and Validation of a Stability-Indicating Reverse Phase HPLC Method for Simultaneous Estimation of Sitagliptin and Metformin in Drug Substance and Combination Formulations

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KEYWORDS

Sitagliptin Phosphate, Metformin HCL, RP-HPLC, ICH Q2R2, Method Validation, LOQ, and LOD establishment.

ABSTRACT:

The quantification of impurities and their degradants in the active pharmaceutical ingredients (APIs) of sitagliptin and metformin, as well as their combinations, is essential for quality control analysis and stability indication for product approval. The method was developed and validated in accordance with the International Conference on Harmonisation (ICH) Q2 R2 guidelines. This sensitive method was employed to detect and quantify impurities in both APIs and finished dosage forms for 50 mg/500 mg, 50 mg/850 mg, and 50 mg/1000 mg tablet formulations. The same samples of the reference formulations were compared with the generic compositions. The analytical method developed for sitagliptin quantification utilized Mobile Phase A, consisting of 0.1% orthophosphoric acid (OPA) as mobile phase-A, and Mobile Phase B, comprising 100% acetonitrile in a gradient composition. This was conducted using a Waters µBondapak C18 250 × 4.6 mm, 5 µm column with a column oven temperature of 50°C, an injection volume of 50 µL, and a flow rate of 1.0 mL/min at 210 nm, employing diluent-1 (acetonitrile and 0.1% OPA 90:10% v/v) and diluent-2 (0.1% OPA solution). Metformin HCL quantification was achieved using a C18 column stationary phase with a mobile phase of potassium dihydrogen phosphate and n-hexane sulfonic acid at pH 3.5 ±, and methanol in a ratio of 95:5% v/v as Mobile Phase A, and 100% acetonitrile as Mobile Phase B, with a flow rate of 0.8 mL/min and an injection volume of 10 µL at a column oven temperature of 30°C, utilizing dual wavelengths of 218 nm and 200 nm. The limit of detection was established at 0.05% relative to the test concentrations, ensuring the method's capability to detect all known and unknown impurities in both analytes within the APIs and the finished dosage forms.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a progressive metabolic disorder marked by insulin resistance, impaired insulin secretion, and increased hepatic glucose production. Due to its multifactorial pathophysiology, effective glycemic management often requires combination therapy that targets various mechanisms of hyperglycemia. Fixed-dose combination (FDC) tablets have emerged as a significant strategy to enhance therapeutic efficacy, simplify treatment regimens, and improve patient adherence. The combination of Sitagliptin and Metformin hydrochloride constitutes a rational and widely prescribed oral antidiabetic therapy. Sitagliptin, a selective dipeptidyl peptidase-4 (DPP-4) inhibitor, enhances endogenous incretin hormones like glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP).[1]This leads to increased glucose-dependent insulin secretion and reduced glucagon release, thereby improving both postprandial and fasting plasma glucose levels with a low risk of hypoglycemia. Metformin hydrochloride, a first-line biguanide agent, primarily reduces hepatic gluconeogenesis and enhances peripheral insulin sensitivity. It also boosts glucose uptake in skeletal muscle and decreases intestinal glucose absorption. Beyond its antihyperglycemic effects, metformin is

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linked to weight neutrality or modest weight loss and demonstrates long-term safety.[2,3] When combined in a single tablet, sitagliptin and metformin HCl offer complementary and synergistic glycemic control by addressing both insulin resistance and impaired incretin function—two core defects in T2DM. Fixed-dose combination tablets not only enhance HbA1c reduction compared to monotherapy but also decrease pill burden, improve compliance, and support sustained metabolic control. Thus, the sitagliptin–metformin combination tablet serves as an effective, convenient, and evidence-based therapeutic option for the comprehensive management of type 2 diabetes mellitus.[4,5]

The Combination Pharmacological Mechanism:

The fixed-dose combination of Sitagliptin and Metformin hydrochloride addresses multiple pathophysiological defects of type 2 diabetes mellitus (T2DM), leading to complementary and synergistic glycaemic control. Sitagliptin is a selective dipeptidyl peptidase-4 (DPP-4) inhibitor. By inhibiting the DPP-4 enzyme, which degrades incretin hormones, it increases the levels of active glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), thereby enhancing glucose-dependent insulin secretion from pancreatic β cells. This process reduces glucagon secretion from α-cells and indirectly lowers the hepatic glucose output.[6] Owing to its glucose-dependent action, sitagliptin carries a low risk of hypoglycaemia. Metformin's mechanism of action primarily involves decreasing hepatic gluconeogenesis (thus reducing liver glucose production), improving peripheral insulin sensitivity in skeletal muscle, increasing glucose uptake and utilization, reducing intestinal glucose absorption, and enhancing insulin receptor activity. Metformin does not stimulate insulin secretion and therefore typically does not cause hypoglycemia.[7]

The synergistic action of the combination is evident, as metformin reduces baseline (fasting) glucose levels by suppressing hepatic glucose production, whereas sitagliptin enhances postprandial glucose control by boosting incretin-mediated insulin release. Both medications decrease glucagon-mediated hepatic glucose output. This combination simultaneously addresses insulin resistance and impaired incretin response, leading to a more significant reduction in HbA1c than monotherapy, with a favourable safety profile and minimal risk of hypoglycaemia. The sitagliptin–metformin fixed-dose combination offers a rational pharmacological approach by targeting complementary mechanisms in T2DM, enhancing incretin activity, improving insulin sensitivity, and reducing hepatic glucose production. Available in both immediate- and extended-release formulations, this combination optimises glycaemic control, improves patient adherence, and maintains a favourable safety profile of the treatment.[8-10]

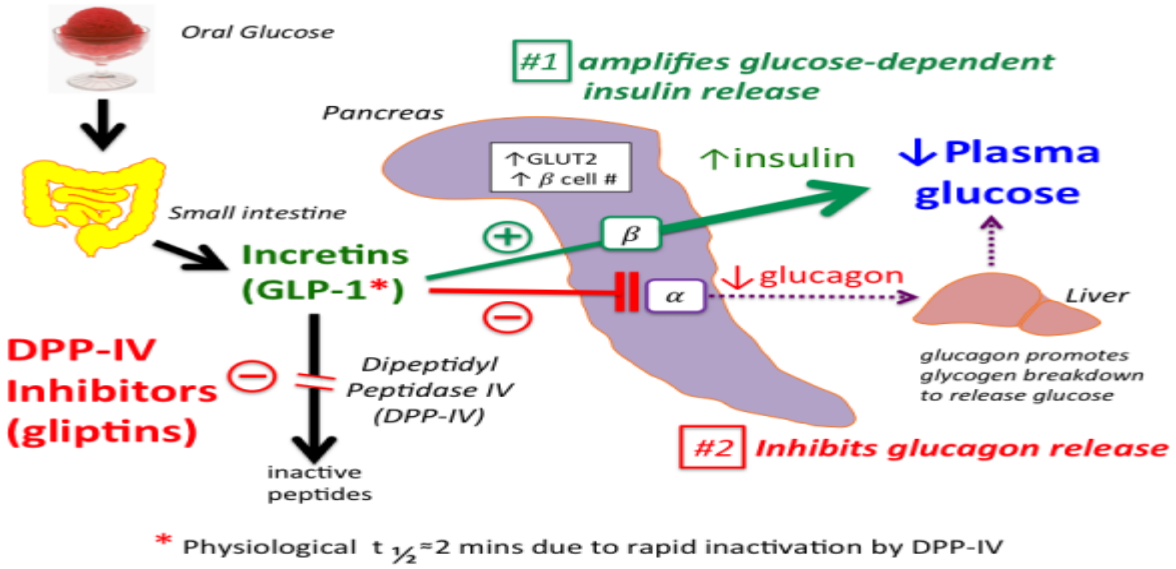

Figure 1: The physiological evaluation of drug actions

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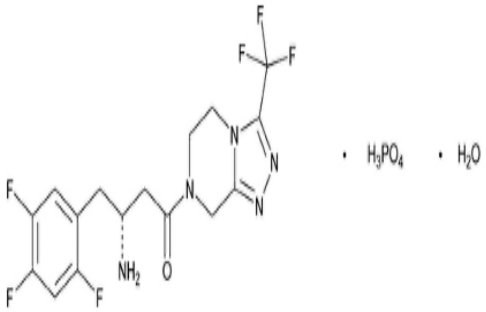
a)



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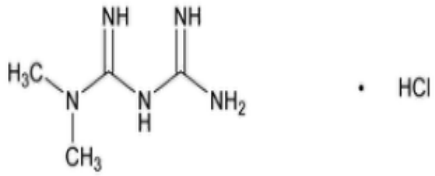

Figure 2: Chemical structures for a) Sitagliptin Phosphate b) Metformin HCL as Per USP

2. Materials and Methods

2.1 Chemicals and Reagents

The reference standards for sitagliptin phosphate anhydrous and metformin HCl were obtained from the USPas reference material. Sitagliptin and metformin tablets in strengths of 50mg/500mg, 50mg/850mg, and 50mg/1000mg were utilized for validation studies. All reagents used were of high analytical purity to minimize background interference in mass spectrometric detection.[11] Methanol, potassium dihydrogen phosphate (ACS grade), 1-Hexane sulphonic acid sodium salt anhydrous (Merck Life Sciences), and orthophosphoric acid (ACS grade) were procured from established commercial sources. High-purity water was generated using a Milli-Q water purification system. PVDF, Nylon membrane and Micro glass fibre filters with a pore size of 0.45 µm were employed for sample filtration, a crucial step often optimized to prevent analyte loss [12].

2.2 Instrumentation and Chromatographic Conditions

The analysis was conducted using a Waters HPLC with Empower-3 software, connecting by UV/PDA detectors. The Chromatographic separation was achieved for sitagliptin won awaters µ Bonda pack (5 µm particle size, 250 x 4.6 mm dimensions) maintained at a constant temperature of 50°C. The mobile phase system consisted of Mobile Phase A, comprising 0.1% OPA in water, and Mobile Phase B, consisting of 100% methanol. [13]

A gradient elution program was implemented to optimize the separation of the impurity from the main peak and matrix components. The flow rate was maintained at 1.0 mL/min with an injection volume of 50 µL. The autosampler temperature was controlled at 25°C to ensure the stability of the sample solutions during the analytical run [14]

The Chromatographic separation was achieved for metformin won a waters symmetry C18 (5 µm particle size, 250 x 4.6 mm dimensions) maintained at a constant temperature of 30°C. The mobile phase system consisted of Mobile Phase A, comprising pH 3.5 buffer: methanol 95:5 %v/v, and Mobile Phase B, consisting of 100% acetonitrile. [15]

A gradient elution program was implemented to optimize the separation of the impurity from the main peak and matrix components. The flow rate was maintained at 0.8 mL/min with an injection volume of 10 µL. The autosampler temperature was controlled at 10°C to ensure the stability of the sample solutions during the analytical run. [15]

Table 1. Chromatographic Conditions for Sitagliptin

Parameter	Condition
Instrument	Waters alliance e2695 equipped with UV/PDA Detectors
Column	Waters µ Bonda pack C18 (250*4.6, 5µm) or equivalent
Column Temperature	50°C
Sample Temperature	25°C

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Flow Rate	1.0 mL/min
Injection Volume	50 µL
Run Time	95 minutes
Mobile Phase A	0.1% OPA in water
Mobile Phase B	Acetonitrile 100%
Diluent-1	Acetonitrile: 0.1% OPA Buffer 90:10 %v/v
Diluent-2	0.1% OPA Buffer 100%
Gradient Program	

Time (Minutes)	**% of Mobile Phase -A**	**% of Mobile Phase -B**
0	95	5
11	95	5
32	85	15
42	80	20
72	70	30
75	70	30
78	30	70
86	30	70
87	94	6
95	94	6

Table 2. Chromatographic Conditions for Metformin

Parameter	**Condition**
Instrument	Waters alliance e2695 equipped with UV/PDA Detectors
Column	Waters Symmetry C18 (250*4.6, 5µm) or equivalent
Column Temperature	30°C
Sample Temperature	25°C
Flow Rate	0.8 mL/min
Injection Volume	10 µL
Run Time	70 minutes
Mobile Phase A	Buffer: 1.36g potassium dihydrogen phosphate into a 1000mL and 1.2g of 1-Hexane sulphonic acid sodium salt anhydrous pH 3.5 with OPA.

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	Mix buffer and methanol in 95:5 % v/v
Mobile Phase B	Acetonitrile 100%
Diluent	12.5mL Acetonitrile in 1000mL Milli-Q water
Gradient Program	

Time (minutes)	% of mobile phase -A	% of mobile phase -B
0	98	2
35	87	13
40	87	13
43	25	75
51	25	75
53	98	2
65	98	2
67	98	2
70	98	2

2.4 Preparation of Standard and Sample Solutions

For sitagliptin, diluent-1 was prepared using acetonitrile and a 0.1% OPA buffer in a 90:10 %v/v ratio for stock preparations, whereas diluent-2, consisting of 0.1% OPA in Milli-Q water, was used for all final preparations. For metformin HCL, 12.5 mL of acetonitrile in 1000 mL of Milli-Q water was used for all preparations. Standard stock solutions of sitagliptin and metformin were prepared in their respective diluents at a concentration of approximately 100 ppm. [16] This solution was serially diluted to obtain a working standard solution with concentrations of 1.24 ppm and 2.5 ppm, corresponding to the specification level. Additionally, system suitability and sensitivity solutions were prepared with sitagliptin at 250 ppm of the sample, 0.5 ppm of sitagliptin fumarate adduct, and 0.124 ppm of sitagliptin sensitivity solutions in diluent-2 for system verification. For the placebo drug substance sample preparation, 10 tablets were weighed and crushed, and approximately 250 mg equivalent of sitagliptin tablet powder was transferred to a 100 mL volumetric flask. Then, 60 mL of diluent-1 was added, and the mixture was sonicated for 60 min for matrix extraction. The volume was made up with diluent-1 and mixed well to obtain the solution at RT.. The final concentration of the sample was 250 ppm in diluent-2, achieved by filtering the solution through a 0.45 µm PVDF filter and discarding 2 mL. For the metformin drug substance sample preparation, 10 tablets were weighed and crushed, and approximately 50 mg of metformin tablet powder was transferred into a 100 mL volumetric flask. Then, 60 mL of diluent was added, and the mixture was sonicated for 30 min. The volume was made up of the diluent and mixed well. The solution was filtered using a 0.45 µm PVDF filter, discarding 2 mL of the solution. The final concentration of the samples was 500 ppm. [17,18]

3. Results and Discussion

3.1 System Suitability and Specificity

The system suitability of the method was assessed by analyzing six replicate injections of the working standard solution. The relative standard deviation (RSD) for the peak area of sitagliptin and metformin was calculated to be 0.2% and 0.3, which is well within the acceptance limit of 5.0%. Moreover, the bracketing standards analyzed throughout the sequence demonstrated recovery values between 100.0% and

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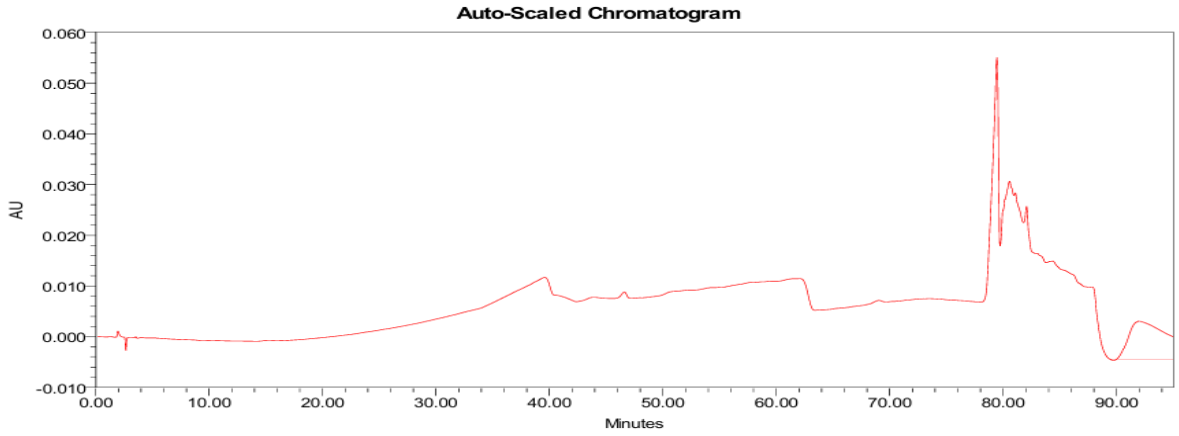
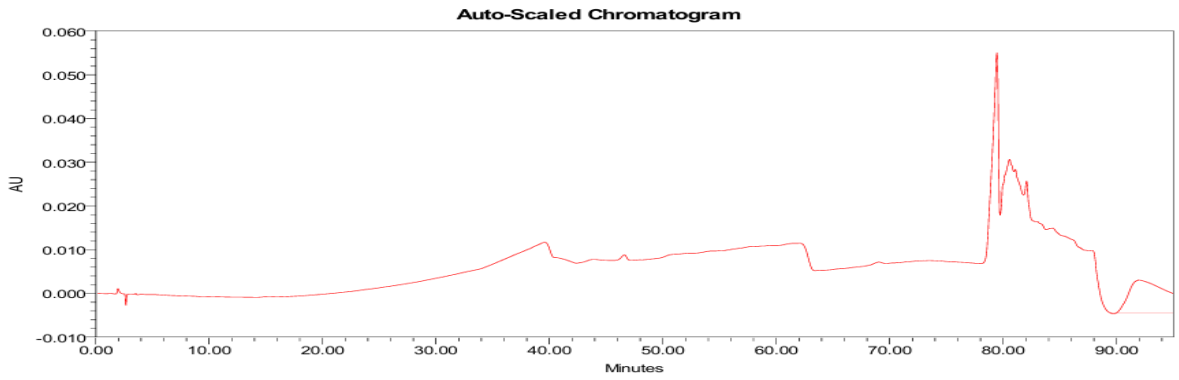
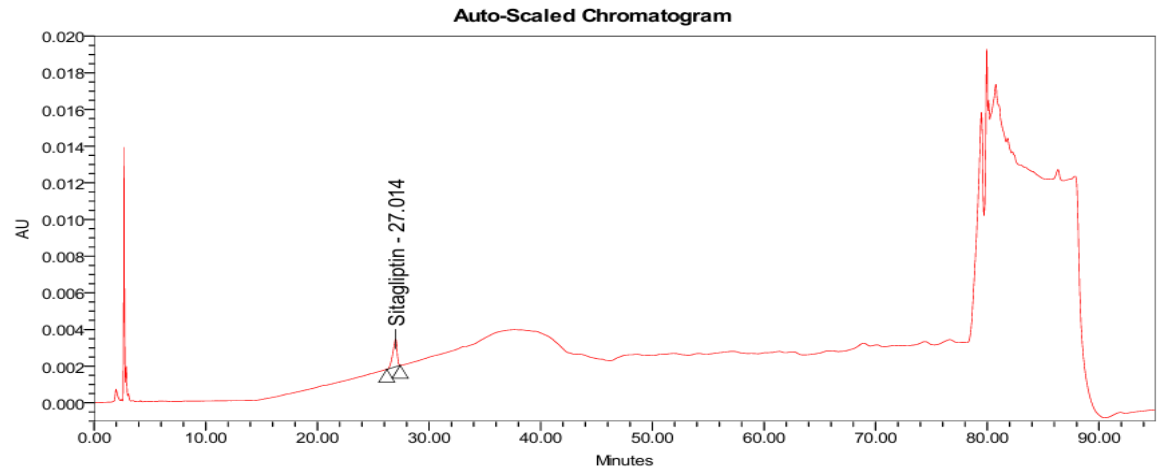
100.1%, confirming the stability of the system during the analysis.

Specificity was evaluated to verify that the method could unequivocally assess the analyte in the presence of components such as degradants, excipients, and the active pharmaceutical ingredient. Chromatograms of the blank diluent, placebo formulations for all strengths, and control samples were acquired. No interfering peaks were observed at the retention time of sitagliptin and Metformin (approximately sitagliptin 27.01 and metformin 15.45 minutes). The results confirmed that the placebo matrix and diluent contributed no interference, showing the high selectivity, which is essential for compliance with USP <621>.







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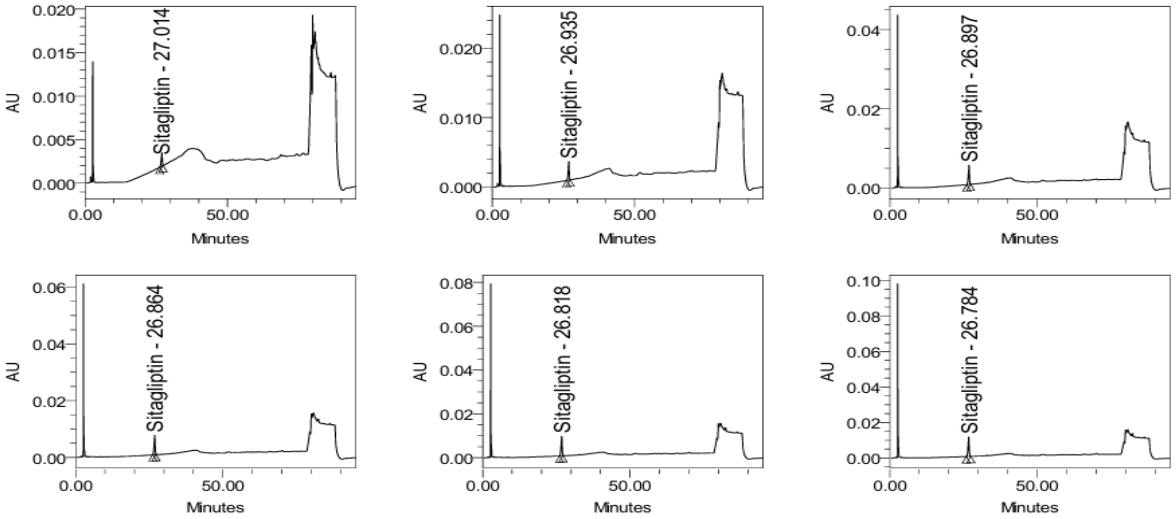
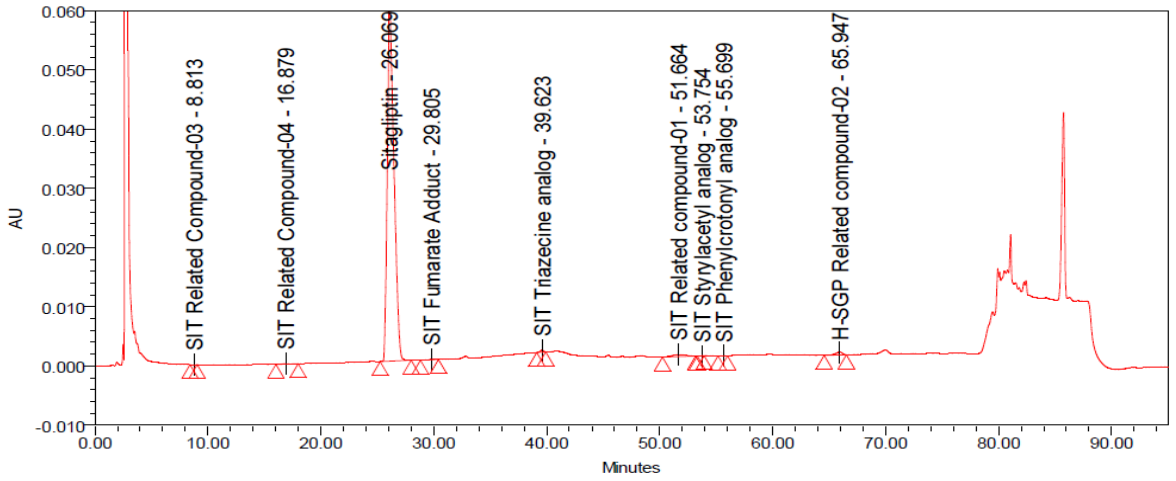
Peak Results

	Name	Retention Time (min)	Area (µV*sec)	s/n	% Area
1	Sitagliptin	27.01	39304	18.5	100



	Peak Name	RT	Area	USP Tailing	USP Plate Count
1	Sitagliptin	26.784	283785	0.9	19675
2	Sitagliptin	26.818	221784	0.8	21128
3	Sitagliptin	26.864	167027	0.8	21989
4	Sitagliptin	26.897	121422	0.8	21786
5	Sitagliptin	26.935	67290	0.8	22483
6	Sitagliptin	27.014	39304	0.7	21500

Auto-Scaled Chromatogram



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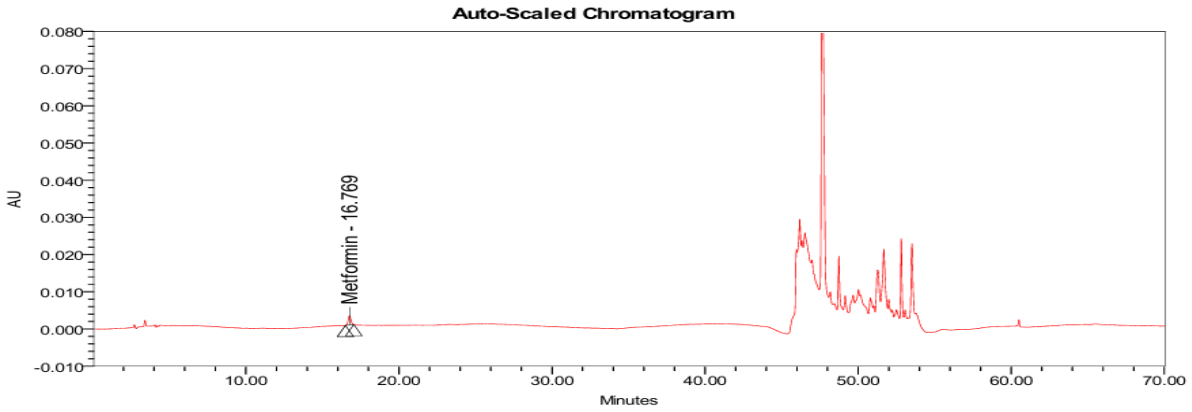
Peak Results

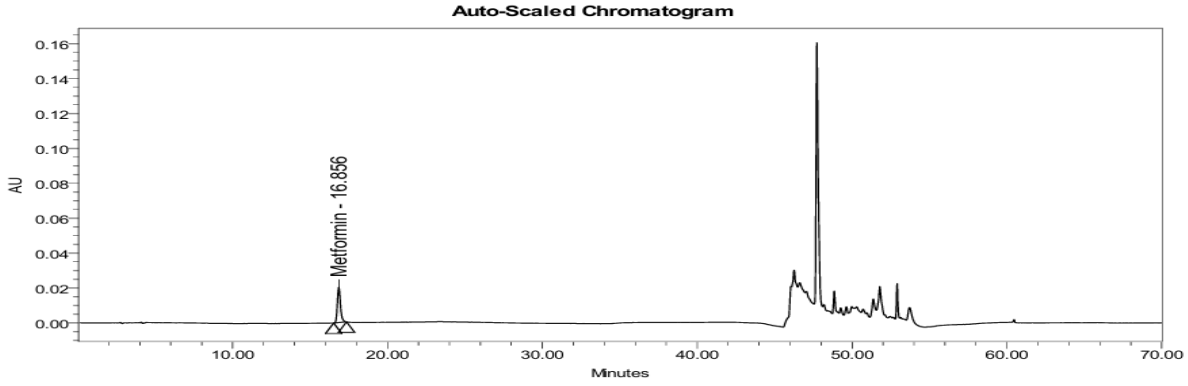
	Name	Retention Time (min)	Area (µV*sec)	% Area	RT Ratio
1	SIT Related Compound-03	8.81	309	0.01	0.34
2	SIT Related Compound-04	16.88	3926	0.16	0.65
3	Sitagliptin	26.07	2465348	97.51	1.00
4	SIT Fumarate Adduct	29.80	2205	0.09	1.14
5	SIT Triazecine analog	39.62	6399	0.25	1.52
6	SIT Related compound-01	51.66	28825	1.14	1.98
7	SIT Styrylacetyl analog	53.75	266	0.01	2.06
8	SIT Phenylcrotonyl analog	55.70	1225	0.05	2.14
9	H-SGP Related compound-02	65.95	19704	0.78	2.53

Figure 3. Typical Chromatograms for Specificity Analysis. Blank Diluent, Placebo, sensitivity and standard solutions and spiked sample for sitagliptin



Peak Results

	Name	Retention Time (min)	Area (µV*sec)	s/n	% Area
1	Metformin	16.77	30649	22.9	100



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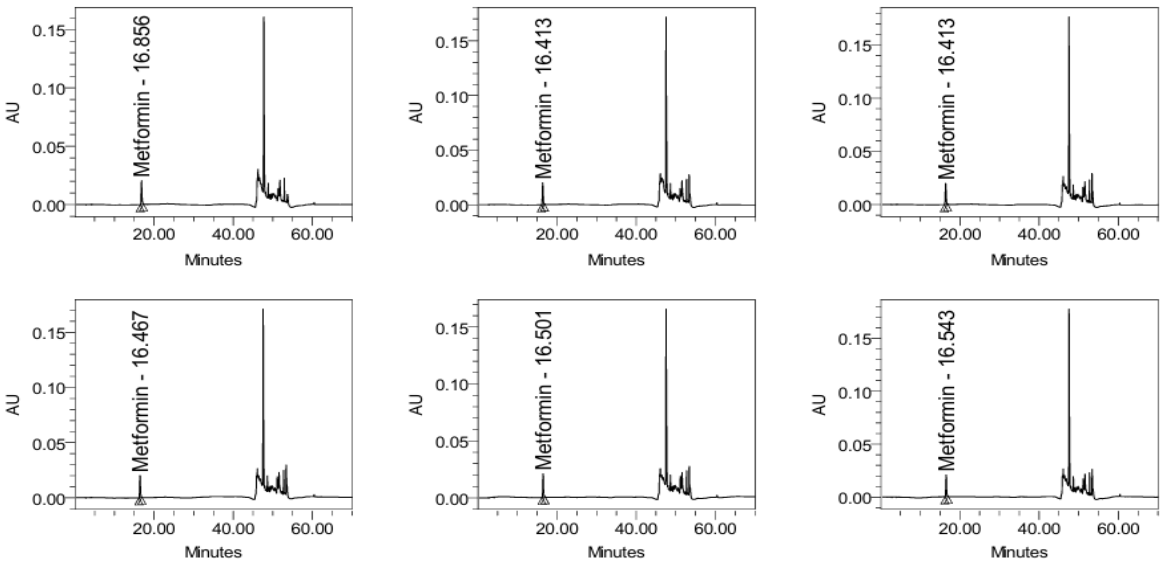
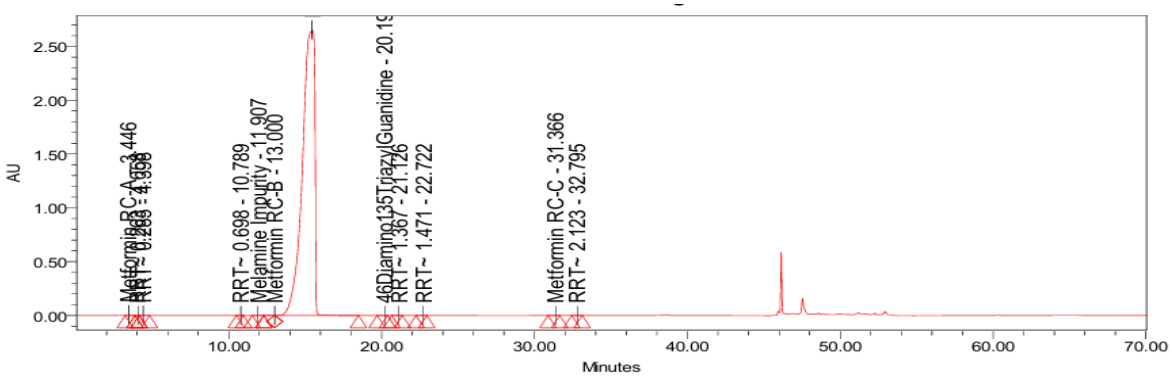
Peak Results

	Name	Retention Time (min)	Area (µV*sec)	USP Tailing	USP Plate Count	Int Type
1	Metformin	16.86	280612	1.2	36171	BB



	Peak Name	RT	Area	USP Tailing	USP Plate Count
1	Metformin	16.413	266129	1.2	36020
2	Metformin	16.413	268608	1.2	35554
3	Metformin	16.467	262567	1.2	37709
4	Metformin	16.501	268947	1.2	38270
5	Metformin	16.543	271075	1.2	38180
6	Metformin	16.856	280612	1.2	36171



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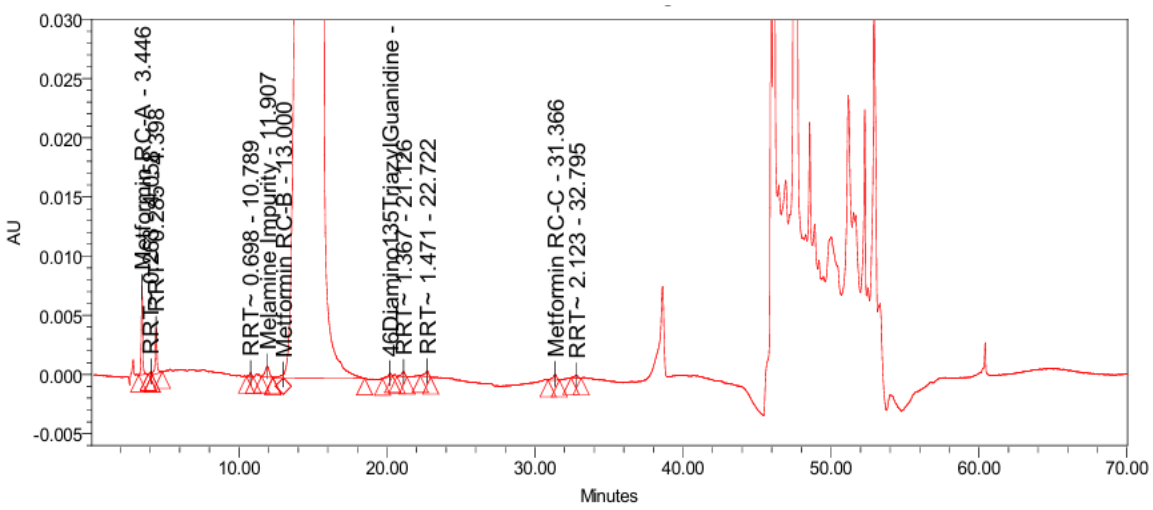

Peak Results

	Name	RT	Area	% Area	Height (µV)	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold	Purity1 Flag	Purity1 Flag
1	Metformin RC-A	3.45	48920	0.03	7460	7034	1.01	2.328	2.848	No	No
2	RRT~ 0.263	4.06	2079	0.00	295	7204	0.74	12.493	17.769	No	No
3	RRT~ 0.285	4.40	30252	0.02	3804	9806	0.82	10.696	5.910	Yes	Yes
4	RRT~ 0.698	10.79	3672	0.00	322	25722	0.79	27.861	32.082	No	No
5	Melamine Impurity	11.91	17031	0.01	901	10530	0.98	3.977	4.735	No	No
6	Metformin RC-B	13.00	4066	0.00	271					No	No
7	Metformin	15.45	148996787	99.91	2653147	2396	0.59	4.463	5.082	No	No
8	46Diamino135TriazylGuanidine	20.20	4357	0.00	298	51515	0.67	11.112	12.834	No	No
9	RRT~ 1.367	21.13	7848	0.01	439	46926	0.74	12.750	13.730	No	No
10	RRT~ 1.471	22.72	9012	0.01	481	34677	0.74	11.524	12.974	No	No
11	Metformin RC-C	31.37	9145	0.01	527	80721	0.76	9.313	8.779	Yes	Yes
12	RRT~ 2.123	32.79	3668	0.00	209	118399	0.87	27.954	29.475	No	No

Figure 4. Typical Chromatograms for Specificity Analysis. Blank Diluent, Placebo, sensitivity and standard solutions and spiked sample for Metformin

3.2 Linearity and Range

The linearity of the analytical method was established by analyzing a series of standard solutions ranging from the Limit of Quantification (LOQ) to 200% of the specification limit.

The concentrations ranges from the 0.070930µg/ml to 0.709330µg/ml of solutions were prepared and injected. And Regression analysis yielded a correlation coefficient (r) of 1.00 and a coefficient of determination (r^2) of 1.00, indicating a perfect linear relationship between concentration and response within the studied range. The slope of the regression line was found to be with in limit and y-intercept of is matched. The residual sum of squares was minimal, further validating the linear model. The method was deemed linear across the range of 20% to 200% of the target specification.

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Table 3: Linearity Results for sitagliptin

Linearity Level	**Concentration (ng/mL)**	**Area Response**	**Statistical Parameters**
Linearity-1 (20%)	0.070930	4974	Slope: 68302.7
Linearity-2 (40%)	0.141870	10212	Intercept: 73.0079
Linearity-3 (70%)	0.248270	15891	Residual Sum of Squares: 2262364
Linearity-4 (100%)	0.354670	23886	Correlation Coefficient (r): 1.00
Linearity-5 (150%)	0.532000	37079	Coefficient of Determination (r^2): 1.00
Linearity-6 (200%)	0.709330	48899	

Table 4: Linearity Results for sitagliptin-related impurities

S.NO	**Name of the Impurity**	**Correlation coefficient**	**% Y intercept**	**RRF**
1	Sitagliptin Acid (H-SGPRC03)	0.9976	2.17	0.60
2	H-SGPRC04	0.9975	3.08	0.52
3	Sitagliptin	0.9991	4.91	----
4	Sitagliptin Fumarate Adduct	0.9992	-5.59	0.55
5	Sitagliptin Triazecine analogue	0.9987	-6.39	1.04
6	H-SGPRC01*	0.9989	-5.55	1.00
7	Sitagliptin Styryl acetyl analogue	0.9995	-1.95	1.84
8	Sitagliptin phenyl crotonyl analogue	0.9993	-2.97	1.67
9	H-SGPRC02*	0.9985	0.85	1.00

Table 5: Linearity Results for Metformin and Metformin Impurity

S.NO	**Name**	**Correlation coefficient**	**%Y-intercept**	**Tentative RRF**
1	Metformin Related Compound-A	0.9999	0.91	2.83
2	Metformin	0.9997	-0.11	-

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3.3 Sensitivity (Limit of Detection and Quantification)

The sensitivity of the method was determined based on the Signal-to-Noise (S/N) ratio approach. The Limit of Quantification (LOQ) was established at 0.0709 ng/mL, which corresponds to 20% of the specification limit. At this concentration, the S/N ratio was found to be greater than 10, and the precision (% RSD) of six replicate injections was 2.0%. The Limit of Detection (LOD) was determined to be 0.038 ng/mL, exhibiting an S/N ratio greater than 3. These values indicate that the method is sufficiently sensitive to quantify sitagliptin and metformin at trace levels required by current regulatory standards.

3.4 Precision and Accuracy

Method precision was evaluated by preparing six independent samples of the drug substance and drug product (0.25 mg and 0.5mg/mL) spiked at the specification level. The % RSD for the drug substance and its impurities was given in the tables below. Intermediate precision was assessed by repeating the analysis on a different day, by a different analyst, and using a different column. The cumulative % RSD (n=12) for the drug product and its related impurities were performed, results were met, showing the robust reproducibility of the method.

Accuracy was determined through recovery studies performed at three concentration levels: LOQ (20%), 100%, and 200% of the specification limit. The study was conducted in triplicate for both the API and the drug product. The mean recovery values for the drug product ranged from 95-105% while the drug substance recoveries ranged from 91.0 to 110.0% for both sitagliptin and metformin. All individual and mean recovery values of all impurities were within the 85.0% to 115.0% acceptance range (70-130% for LOQ), confirming the method's accuracy and extraction efficiency.

Table 6: Precision results for sitagliptin impurities(100% Level)

S.No	Sample No	%w/w of Sitagliptin Acid (H-SGPRCO3)	%w/w of Sitagliptin H SGPRCO4)	%w/w of Sitagliptin Fumarate Adduct
1	Spiked sample pre-01	0.267	0.226	0.193
2	Spiked sample pre-02	0.253	0.198	0.189
3	Spiked sample pre-03	0.252	0.215	0.182
4	Spiked sample pre-04	0.258	0.198	0.181
5	Spiked sample pre-05	0.256	0.202	0.179
6	Spiked sample pre-06	0.258	0.194	0.179
	Average	0.257	0.210	0.184
	%RSD	2.10	6.02	3.15

Table 7: Precision results for sitagliptin impurities (100% Level)

S.No	Sample No	%w/w of Sitagliptin Triazecine analogue (Imp-B)	%w/w of Sitagliptin phenyl crotonyl analogue	%w/w of Sitagliptin Styryl acetyl analogue
1	Spiked sample Pre-01	0.210	0.170	0.195

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S.No	Sample No	%w/w of Sitagliptin Triazecine analogue (Imp-B)	%w/w of Sitagliptin phenyl crotonyl analogue	%w/w of Sitagliptin Styryl acetyl analogue
2	Spiked sample Pre-02	0.213	0.169	0.184
3	Spiked sample Pre-03	0.218	0.168	0.191
4	Spiked sample Pre-04	0.211	0.164	0.186
5	Spiked sample Pre-05	0.218	0.167	0.184
6	Spiked sample Pre-06	0.215	0.167	0.184
	Average	0.214	0.168	0.187
	%RSD	1.59	1.25	2.46

Table 8: % recovery results for sitagliptin impurities (LOQ Level)

S.No	Sample No	%w/w of Sitagliptin Acid (H-SGPRCO3)	%w/w of Sitagliptin H SGPRCO4)	%w/w of Sitagliptin Fumarate Adduct
1	LOQ Precision-01	94.2	91.6	103.6
2	LOQ Precision-02	108.3	102.4	99.5
3	LOQ Precision-03	107.1	98.0	103.6
4	LOQ Precision-04	109.5	105.5	105.6
5	LOQ Precision-05	106.7	105.4	103.1
6	LOQ Precision-06	108.8	110.5	104.6
	Average	105.8	102.9	103.3
	%RSD	5.45	6.49	2.01

Table 9: Recovery results for sitagliptin impurities (LOQ Level)

S.No	Sample No	%w/w of Sitagliptin Triazecine analogue (Imp-B)	%w/w of Sitagliptin phenyl crotonyl analogue	%w/w of Sitagliptin Styryl acetyl analogue
1	LOQ Precision-01	89.8	102.6	93.8
2	LOQ Precision-02	93.2	102.0	91.3
3	LOQ Precision-03	88.6	102.0	88.1

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S.No	Sample No	%w/w of Sitagliptin Triazecine analogue (Imp-B)	%w/w of Sitagliptin phenyl crotonyl analogue	%w/w of Sitagliptin Styryl acetyl analogue
4	LOQ Precision-04	86.9	103.6	93.1
5	LOQ Precision-05	84.7	102.0	88.8
6	LOQ Precision-06	88.6	103.1	86.3
	Average	88.6	102.6	90.2
	%RSD	3.22	0.66	3.29

Table 10: Precision results for Metformin Impurity

S.NO	Sample No.	%W/W of Metformin Related Compound-A
1	Spiked Sample-1	0.161
2	Spiked Sample-2	0.160
3	Spiked Sample-3	0.158
4	Spiked Sample-4	0.160
5	Spiked Sample-5	0.158
6	Spiked Sample-6	0.160
	Average	0.160
	% RSD	0.75

Table 11: Recovery results for Metformin Impurity

S.NO	Accuracy Level	%Recovery	Mean Recovery	Overall Mean Recovery
1	LOQ	89.1	90.6	95.9
2		90.1		
3		88.6		
4		91.1		
5		94.6		
6		90.1		

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3.5 Forced degradation study:

For sitagliptin:

Acid sample preparation (1N HCl at Bechtop-60mins)

Weigh and crush not less than 10 tablets.Accurately weigh and transfer the tablet powder equivalent to 250mg of Sitagliptin into a 100 mL volumetric flask. Add about 60mL of diluent-1 and sonicate for 60 minutes with intermediate shaking (maintain the sonicator temperature between 20-25°C). added 10ml of 1N HCl and kept the flask on the bench top for 60 minutes, and cooled to room temperature and neutralised with 10 ml of 1N NaOH. Dilute to volume with diluent-1 and mix. Centrifuge a portion of the solution at 4500 RPM for 10 minutes.

Transfer 5mL of the above solution into 50 mL volumetric flasks, dilute to volume with diluent-2 and mix. Filter the solution through a 0.45µm PVDF syringe filter and discard the first 3mL of the filtrate.

Base sample preparation (5N Methanolic NAOH at Benchtop-3days):

Weigh and crush not less than 10 tablets. Accurately weigh and transfer the tablet powder equivalent to 250mg of sitagliptin into a 100mL volumetric flask. Add about 60 mL of diluent-1 and sonicate for 60 minutes with intermediate shaking (maintain the sonicator temperature between 20-25°C). added 10ml of 5N Methanolic NaOH and kept the flask on the bench top for 3days, and cooled to room temperature and neutralised with 10 ml of 5N Methanolic HCl. Dilute to volume with diluent-1 and mix. Centrifuge a portion of the solution at 4500 RPM for 10 minutes.

Transfer 5mL of the above solution into a 50mL volumetric flask, dilute to volume with diluent-2 and mix. Filter the solution through a 0.45µm PVDF syringe filter and discard the first 3mL of the filtrate.

Peroxide sample Preparation (1% H2O2 Bench top Intermediate):

Weight and crush not less than 10 tablets. Accurately weigh and transfer powder equivalent to 250mg of sitagliptin into a 100mL volumetric flask, add about 60mL of diluent-1 and sonicate for 60 minutes with intermediate shaking (maintain the sonicator bath temperature between 20-25ºC). Added 5 mL of 1% H202 and immediate preparation. Dilute to volume with diluent-1 and mix.Centrifuge a portion of the solution at 4500 RPM for 10 minutes.

Transfer 5mL of the above solution into a 50 mL volumetric flask, dilute to volume with diluent-2 and mix. Filter the solution through a 0.45µm PVDF syringe filter and discard the first 3mL of the filtrate.

Table 12: Forced degradation results for Sitagliptin

S.NO	Condition	Total Impurities %w/w	%Assay	Mass balance
1	Control sample	0.024	96.9	-
2	1N HCl-60mins at Benchtop	0.244	97.2	100.21
3	5N Methane NAOH-3days at	3.863	95.2	101.98

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	BT			
4	1%H202-immediate	0.030	99.2	102.00
5	Thermal-24hours at 60ºC	0.264	100.9	104.00

For Metformin:

Acid sample preparation (5N HCL at 60mins at 60º C)

Weight and crush not less than 10 tablets. Accurately weigh and transfer the tablet powder. Equivalent to 50mg of Metformin Hydrochloride into a 100mL volumetric flask, add about 60mL of diluent and sonicate for 30minutes with intermediate shaking (maintain the sonicator bath temperature between 20-25ºC). Added 10ml of N HCl and kept the flask in the oven at 60º C for 60 minutes, and cooled to room temperature and neutralized with 10ml of 5N NaOH. Dilute to volume with diluents and mix. Filter the solution through a 0.45µm membrane filter and discard the first 3Ml of the filtrate.

Base sample preparation (2NaOH at 60mins at 60ºC)

Weigh and crush not less than 10 tablets.Accurately weight and transfer the tablet powder equivalent to 50mg of Metformin Hydrochloride into a 100mL volumetric flask, add about 60mL of diluent and sonicate for 30minutes with intermediate shaking (maintain the sonicator bath temperature between 20-25ºC).added 10ml of 2N NAOH and kept the flask on oven at 60ºC for 60mins, and cooled to room temperature and neutralized with 10ml of 2N HCl Diluent to volume with diluent and mix. Filter the solution through a 0.45µm member filter and discard the first 3mL of the filtrate.

Peroxide sample preparation (30% H202 at 60mins at 60ºC):

Weight and crush not less than 10 tablets. Accurately weigh and transfer powder equivalent to 50mg of Metformin Hydrochloride into a 100mL volumetric flask, add about 60mL of diluent and sonicate for 30 minutes with intermediate shaking (maintain the sonicator bath temperature between 20-25ºC). added 10ml of 30% H202 and kept the flask oven at 60ºC for 60mins, and cooled to room temperature. Dilute to volume with diluent and mix well. Filter the solution through a 0.45µm membrane filter and discard the first 3mL of filtrate.

Table 13: Forced degradation results for Metfromin

S.NO	Condition	Total impurities %w/w	% Assay	Mass balance
1	Control Sample	0.016	100.1	-
2	5N HCL-60mins at 60ºC	0.264	96.3	96.5
3	2N NAOH-60mins at60ºC	1.530	95.9	97.3
4	30% H202-60mins at 60ºC	0.531	96.0	96.4

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5	Thermal-24hours at 80ºC	0.015	97.1	97.0

Robustness and Solution Stability

The robustness of the method was challenged by deliberately varying critical parameters, including flow rate (± 0.02 mL/min) and column temperature (± 5°C). Under all tested conditions, the system suitability criteria were met, with the % RSD of standard injections remaining below 5.0% (specifically between 1.0% and 4.7%), and the retention times varied predictably as shown in Table 5. This indicates that the method is reliable and unaffected by small, deliberate variations in operating parameters.

Table 14: Robustness Results for sitagliptin

Variation Condition	% RSD (n=6)	Retention Time (min)
Control	1.0%	27.39
Low Flow (-0.02 mL/min)	2.0%	29.99
High Flow (+0.02 mL/min)	2.1%	25.21
Low Temperature (-5°C)	2.8%	29.35
High Temperature (+5°C)	1.0%	25.92

Note: Acceptance criterion for %RSD is NMT 15.0%.

Solution stability was assessed by storing standard and sample solutions under refrigerated conditions (2-8°C). Re-analysis at intervals up to 48 hours (Day 2) showed that the response did not deviate significantly from the initial analysis. As detailed in Tables 15 and 16, the difference in response remained within 10% for standards and within 10% for drug product samples, well within the 20% acceptance limit.

Table 15: Solution Stability Results for sitagliptin (Refrigerated)

Solution Type	Time Point	% Initial / % Recovery	% Difference from Initial
Standard	Initial	100.0%	N/A
	Day-1	96.7%	3%
	Day-2	96.4%	4%
Drug Substance	Initial	87.6%	N/A
	Day-1	82.7%	6%
	Day-2	80.3%	8%
Drug Product	Initial	87.6%	N/A
	Day-1	82.9%	5%
	Day-2	81.9%	7%

Table 16: Solution Stability Results for Metformin (Refrigerated)

Solution Type	Time Point	% Initial / % Recovery	% Difference from Initial
Standard	Initial	100.2%	N/A
	Day-1	99.7%	2%
	Day-2	99.4%	1%

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Drug Substance	Initial	100.6%	N/A
	Day-1	100.7%	0.1%
	Day-2	100.3%	0.2%
Drug Product	Initial	100.6%	N/A
	Day-1	100.9%	0.3%
	Day-2	100.9%	0.6%

4. Conclusion

A sensitive, specific, and robust RP-HPLC method was successfully developed and validated for the quantification of sitagliptin and metformin-related impurities at the LOQ level to 200 % level for the targeted concentration and drug substance and tablet formulations. The method demonstrates excellent linearity, precision, and accuracy across the validation range of 0.0709 ng/mL to 0.7093 ng/mL.The LOQ of 0.0709 ng/mL allows for the quantification of these unknown impuritieswell below the quantification limits. The validation data confirms that the method complies with ICH Q2(R1) and ICH Q2(R2) guidelines and is suitable for routine quality control applications to ensure the safety and quality of Trimipramine Maleate pharmaceutical products.

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