RESEARCH PAPER

Method Development and Validation for Related Impurities of Sitagliptin by RP-HPLC Method

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ABSTRACT

Sitagliptin, an oral antidiabetic agent used in the management of type 2 diabetes mellitus, requires stringent quality control to ensure its safety and efficacy. The present study focuses on the development and validation of a simple, precise, and reliable Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method for the determination of related impurities in Sitagliptin. Chromatographic separation was achieved using a suitable C18 column with an optimized mobile phase composed of buffer and organic solvent under gradient/isocratic conditions. The detection was carried out using a UV detector at an appropriate wavelength. The method was validated in accordance with International Council for Harmonisation (ICH) guidelines with respect to specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. The developed method demonstrated good resolution between Sitagliptin and its related impurities, with acceptable system suitability parameters. Linearity was observed over a suitable concentration range with high correlation coefficients. The recovery studies confirmed the accuracy of the method, while precision studies indicated reproducibility with low %RSD values. The validated RP-HPLC method was found to be sensitive, specific, and reliable for routine analysis of Sitagliptin and its related impurities in bulk and pharmaceutical dosage forms. Hence, the method can be effectively employed for quality control and stability studies in pharmaceutical industries.

Key Words: C18 column, RSD, ICH, LOQ, RP-HPLC

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Introduction:

Sitagliptin is a widely used oral hypoglycemic agent belonging to the class of dipeptidyl peptidase-4 (DPP-4) inhibitors, indicated for the treatment of type 2 diabetes mellitus. It acts by inhibiting the DPP-4 enzyme, thereby increasing incretin levels, which in turn enhance insulin secretion and reduce glucagon levels in a glucose-dependent manner. Due to its therapeutic importance and widespread use, maintaining the quality, safety, and efficacy of Sitagliptin is essential.

During the synthesis, storage, and formulation of pharmaceutical drug substances and products, impurities may be formed. These impurities can arise from starting materials, intermediates, degradation products, or side reactions. The presence of such related impurities, even in trace amounts, may affect the safety and stability of the drug. Therefore, identification, separation, and quantification of impurities are critical components of pharmaceutical analysis and are strictly regulated according to International Council for Harmonisation (ICH) guidelines such as Q3A and Q3B.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) is one of the most widely employed analytical techniques for the determination of pharmaceutical compounds and their impurities due to its high sensitivity, accuracy, and reproducibility. It offers efficient separation of compounds based on their polarity and is highly suitable for routine quality control analysis.

The present study aims to develop and validate a simple, rapid, and robust RP-HPLC method for the determination of related impurities in Sitagliptin. The developed method is validated as per ICH guidelines with respect to parameters such as specificity, linearity, precision, accuracy, robustness, limit of detection (LOD), and limit of quantification (LOQ). This method

can be effectively applied for routine quality control and stability testing of Sitagliptin in bulk and pharmaceutical dosage forms.

Materials and Methods:

Chemicals and Reagents

Sitagliptin working standard was obtained from a certified pharmaceutical source. HPLC grade solvents such as methanol, acetonitrile, and water were used throughout the study. Analytical grade reagents including potassium dihydrogen phosphate and orthophosphoric acid were used for the preparation of buffer solutions. All chemicals and reagents were of suitable analytical or HPLC grade.

Instrumentation

The analysis was performed using an RP-HPLC system equipped with a quaternary pump, auto-sampler, column oven, and UV detector. Data acquisition and processing were carried out using appropriate chromatographic software.

Chromatographic Conditions

The chromatographic analysis was carried out using a mobile phase consisting of methanol and 0.1% orthophosphoric acid in water (80: 20, % *v/v*). The detection wavelength was set at 266 nm to ensure optimal sensitivity for the analyte. The mobile phase was delivered at a flow rate of 1.0 mL/min, and the column temperature was maintained at 25°C to ensure consistent chromatographic performance. A sample volume of 20 µL was injected for each run.

Experimental Work

Preparation of stock standard solution and **w**orking solution (Stock-I)

A precisely weighed amount of 10 mg of SITA standard was transferred into a clean, dry 10 mL volumetric flask. Methanol was added to dissolve the standard, and the solution was sonicated if necessary to ensure complete dissolution. The volume was then made up to the 10 mL mark with methanol, resulting in a stock solution with a final concentration of 1000 µg/mL (Stock-I). This primary stock solution was stored in an amber vial to protect it from light and used for further dilutions during method development and analysis.

Preparation of Impurity Sample Solution

For Impurity I

For impurity I (IMP-1), an accurately weighed quantity of 5 mg was transferred into a clean, dry 10 mL volumetric flask. Methanol was added to dissolve the weighed material, and the solution was gently mixed and sonicated, if necessary, to achieve complete dissolution. The volume in each flask was then brought up to the 10 mL mark with methanol, resulting in individual stock solutions with a concentration of 500 µg/mL for Impurity.

For Impurity II

For the preparation of the impurity stock solution, accurately weighed quantities of Impurity-1 and Impurity-2 (IMP-2) (5 mg each) were transferred into separate clean and dry 10 mL volumetric flasks. Methanol was added to each flask to dissolve the impurities, and the solutions were gently mixed and sonicated, if required, to ensure complete dissolution. The volume in each flask was then made up to the 10 mL mark with methanol, yielding stock solutions with a concentration of 500 µg/mL for IMP-1 and IMP-2, respectively. These preparations were designated as Stock-III solutions and were stored appropriately until further dilution and analysis.

Preparation of working standard solutions

To prepare the mixed working solutions used in the study, graded concentrations of SITA were combined with fixed levels of IMP-1 and IMP-2. The process began by taking 0.1 mL of the SITA Stock-I solution together with 0.2 mL of the impurity Stock-III solution and diluting the mixture to 10 mL with the mobile phase, giving a final concentration of 5 µg/mL of SITA along with 10 µg/mL each of IMP-1 and IMP-2. In the same manner, additional working solutions were prepared by increasing the volume of Stock-I to 0.2 mL, 0.3 mL, 0.4 mL, and 0.5 mL, respectively, while keeping the impurity volume constant at 0.2 mL. Each mixture was then made up to 10 mL with the mobile phase. These preparations resulted in solutions containing 10, 15, 20, and 25 µg/mL of SITA, each consistently accompanied by 10 µg/mL of both impurities. All solutions were thoroughly mixed to ensure proper homogenization before use in calibration and analytical evaluation.

Preparation of sample solution

Preparation of Sample Stock Solution (Stock-II)

The marketed formulation selected for analysis was RIFAMET (400 mg). The combined weight of twenty tablets was found to be 11.11 g, giving an average tablet weight of 555.6 mg. Based on this average weight and the label claim of 400 mg of SITA per tablet, the amount of tablet powder equivalent to 10 mg of SITA was calculated. From this calculation, 13.89

mg of the finely powdered tablet blend was accurately weighed and transferred into a clean 100 mL volumetric flask. Methanol was then added to dissolve the sample, and the volume was made up to the mark. This procedure yielded a sample stock solution containing 100 µg/mL of SITA, which was designated as Stock-II and used for further analytical work.

Tablet Assay Preparation

For the assay of the tablet formulation, the powdered tablet sample was first extracted to obtain Sample Stock-I, followed by appropriate dilution to prepare Sample Stock-II. From this intermediate stock solution, 4 mL from Stock-II was accurately transferred to a 10 mL volumetric flask and the volume was made up with the mobile phase, yielding a final working concentration of 40 µg/mL of SITA. The solution was mixed thoroughly to ensure uniformity and clarity before analysis. This final assay solution was then injected into the HPLC system under the optimized chromatographic conditions, and the peak area obtained was compared with that of the standard solution to determine the amount of SITA present in the tablet formulation. This procedure ensured accurate dilution, appropriate sample handling, and reproducible quantification suitable for routine quality-control evaluation.

Results and Discussion:

Method Development

In the optimized conditions it has been observed that the SITA, IMP-1 and IMP-2 were well separated with a resolution greater than 4. Peak symmetry was assessed using the asymmetry factor. All peaks exhibited acceptable symmetry.

Table 1: System Suitability Parameters for Optimized Chromatographic Condition

Name	Retention Time (min)	Area	Resolution	No. of Theoretical plates
SITA	2.278	216.07245	-	5443
IMP-1	3.874	9.65691	11.30	9532
IMP-2	4.643	26.91240	4.52	10538

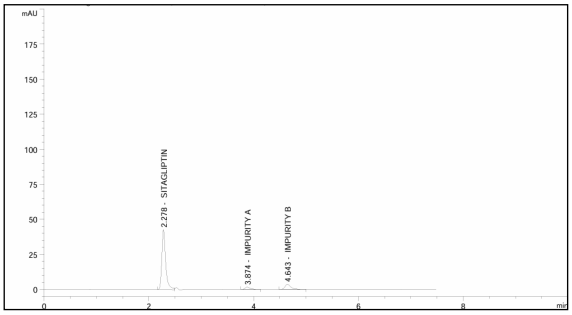


Figure 1: Chromatogram showing the separation of SITA, IMP-1 and IMP-2

Tablet Assay Preparation

For the assay of the tablet formulation, an appropriate working solution was prepared from the previously obtained sample Stock-II solution. From this stock, 4 mL was accurately transferred into a 10 mL volumetric flask and diluted to volume with the mobile phase. This dilution produced a final concentration of 40 µg/mL of SITA, which was used for the quantitative assay of the tablet sample. The solution was mixed thoroughly to ensure uniformity before injection into the HPLC system.

Table 2: Analysis of Drug from Tablet Formulation

Conc.	Peak Area-I	Peak Area-II	Mean Peak Area	SD	% RSD
40.00 µg/mL	290.88	289.06	289.97	1.287	0.444

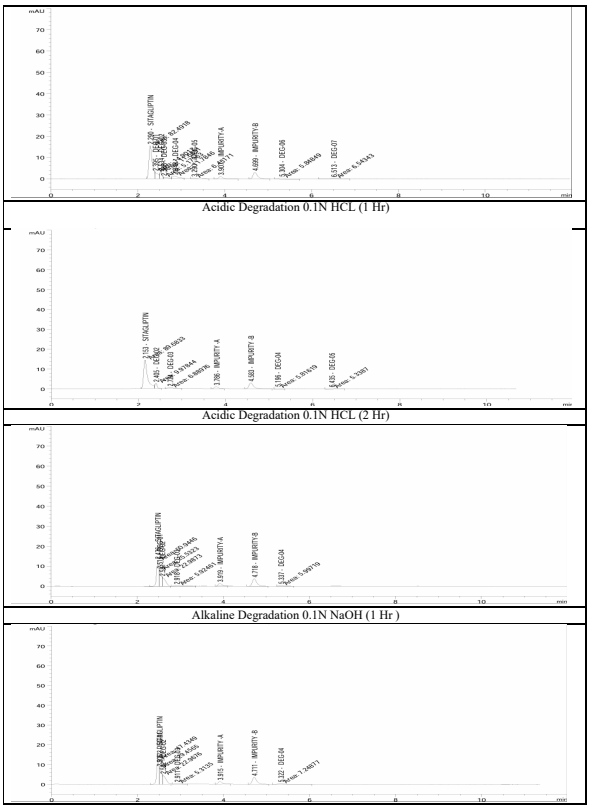
Method Validation

Specificity

Forced degradation data

		SITA			
Sr. No.	**Degradation**	**Area of Standard**	**Area of degraded Sample**	**Degraded upto %**	**Actual % degradation**
		AFTER 1 HR			
1	Acid Degradation	97.5666	82.4918	84.55	15.45
2	Basic Degradation	97.5666	80.9446	82.96	17.04
3	H_2O_2 Degradation	97.5666	89.3173	91.54	8.46

4	Neutral	97.5666	87.5666	89.75	10.25
AFTER 2 HR					
1	Acid Degradation	97.5666	79.6833	81.67	18.33
2	Basic Degradation	97.5666	47.1349	48.31	51.69
3	H_2O_2 Degradation	97.5666	69.6112	71.35	28.65
4	Neutral	97.5666	-	-	-



Linearity and Range

Compound	Linearity Range (µg/mL)	Correlation Coefficient (R^2)	Slope	Y-Intercept
SITA	2.00-10.00	0.9993	19.838	18.629

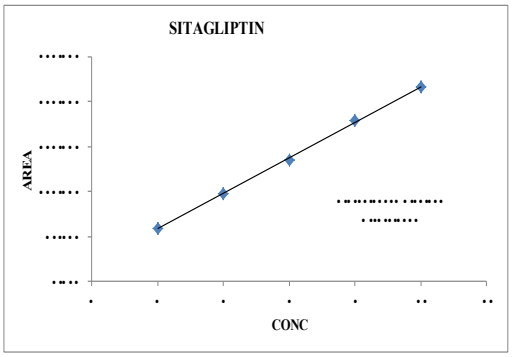


Figure 3: Linearity assessment of SITA

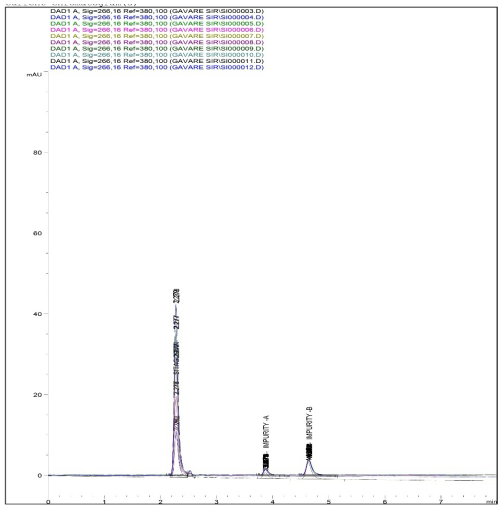


Figure 4: Typical overlay chromatogram of linearity

Precision

Precision of the method was evaluated by analyzing SITA at three concentration levels (2, 6, and 10 µg/mL) in the presence of fixed concentrations of IMP-1 and IMP-2 (10 µg/mL each), with duplicate injections at each level. In the intraday precision study, the % RSD ranged from 0.43 to 2.42 for SITA, 0.42 to 3.25 for IMP-1, and 1.22 to 2.53 for IMP-2. Similarly, in the Interday precision study, the % RSD ranged from 0.10 to 0.58 for SITA, 0.13 to 12.21 for IMP-1, and 0.36 to 1.32 for IMP-2. The method demonstrated good precision, with % RSD values for both intraday and Interday studies found to be below 2%.

Intra-day precision

Sample	Conc. (µg/ml)	Peak Area 1	Peak Area 2	Mean	SD	%RSD
SITA	2	59.63253	57.6262	58.63	1.42	2.42
	6	136.6146	135.7828	136.20	0.59	0.43

	10	216.519	214.8142	215.67	1.21	0.56
IMP-1	10	9.09681	9.15146	9.12	0.04	0.42
	10	10.44788	9.97906	10.21	0.33	3.25
	10	10.13918	9.94225	10.04	0.14	1.39
IMP-2	10	27.87418	28.46489	28.17	0.42	1.48
	10	29.06408	30.12269	29.59	0.75	2.53
	10	26.19273	25.74348	25.97	0.32	1.22

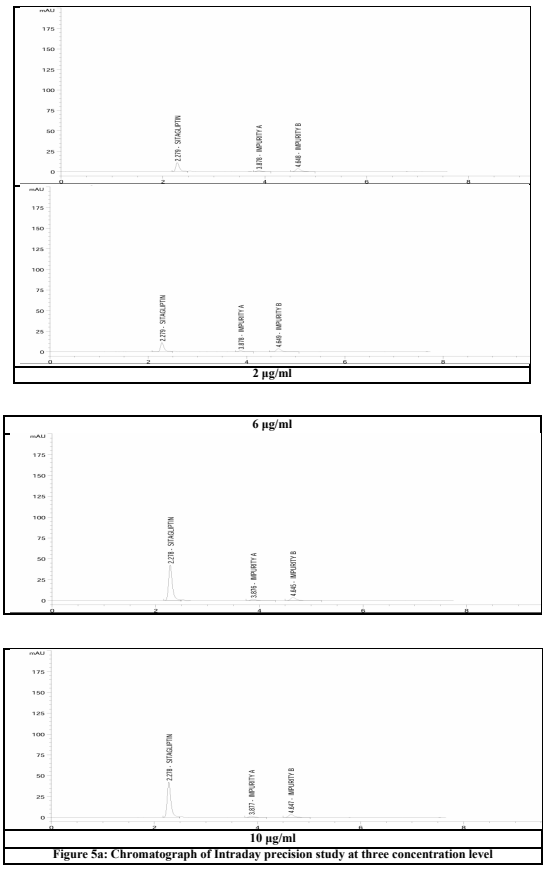


Figure 5a: Chromatograph of Intraday precision study at three concentration level

Accuracy

For the accuracy study, a 10 µg/mL tablet solution was used as the base concentration. From this, three accuracy levels 80%, 100%, and 120% were prepared to evaluate the reliability and recovery of the method. To prepare the 80% level, 0.5 mL of the tablet solution was transferred into a 10 mL volumetric flask, followed by the addition of 0.08 mL of the standard solution; the mixture was then diluted to volume with the mobile phase to obtain a final concentration of 4 µg/mL. For the 100% level, 0.5 mL of the tablet solution and 0.1 mL of the standard solution were combined in a 10 mL flask and diluted to the mark, yielding a 5 µg/mL solution. Similarly, the 120% level was prepared by adding 0.5 mL of the tablet solution and 0.12 mL of the standard solution into a 10 mL volumetric flask and making up the volume with the mobile phase to obtain a final concentration of 6 µg/mL. All solutions were mixed thoroughly to ensure homogeneity before analysis.

Accuracy for IMP-1 and IMP-2 was evaluated by recovery studies at three levels corresponding to 80%, 100%, and 120% of the nominal drug concentration while maintaining a constant impurity spike level of 10 µg/mL. Each level was prepared in triplicate and analyzed as per the test method. Percent recovery was calculated by comparing the measured impurity content against the known spiked amount.

Recovery results of IMP-1

Level (%)	% Recovery	Mean % Recovery	SD	% RSD
80	98.2	99.57	1.31	1.31
	100.8			
	99.7			
100	100.3	100.7	1.44	1.43
	99.5			
	102.3			
120	101	101.4	1.83	1.81
	99.8			
	103.4			

Recovery results of IMP-2

Level (%)	% Recovery	Mean % Recovery	SD	% RSD
80	98.2	99.93	1.80	1.80
	99.8			
	101.8			
100	99.7	101.17	1.45	1.43
	101.2			
	102.6			
120	99.2	100.9	1.81	1.79
	102.8			
	100.7			

Robustness

The robustness study was performed to evaluate the reliability of the analytical method under small, deliberate variations in chromatographic conditions. Changes in mobile phase composition, wavelength, and flow rate were assessed using a standard

concentration of 8 µg/mL, and results were compared in terms of peak area, mean, SD, and % RSD, and the detailed results are tabulated in **Table** for SITA, IMP-1, and IMP-2.

Robustness evaluation

Peak Name	Change in Parameter	Modified condition	Peak area 1	Peak Area 2	Mean peak area	SD	%RSD
SITA	Mobile phase composition (Methanol: 0.1% orthophosphoric acid in water)	79:21	185.18	188.70	186.94	2.49	1.33
		81:19	183.47	182.67	183.08	0.57	0.31
IMP-1		79:21	9.57923	9.00865	9.29	0.40	4.34
		81:19	9.29546	9.84583	9.57	0.39	4.07
IMP-2		79:21	26.46384	26.10846	26.29	0.25	0.96
		81:19	24.71104	24.45068	24.58	0.18	0.75
SITA	Wavelength	265	186.42	185.96	186.2	0.33	0.18
		267	200.77	198.44	199.61	1.65	0.82
IMP-1		265	10.59065	10.28392	10.4	0.22	2.08
		267	10.90564	11.34179	11.12	0.31	2.77
IMP-2		265	27.97432	27.95097	28.0	0.02	0.06
		267	26.66721	25.49601	26.08	0.83	3.18
SITA	Flow Rate	0.9 mL	198.3496	199.4304	198.89	0.76	0.38
		1.1 mL	165.3332	162.2783	163.81	2.16	1.32
IMP-1		0.9 mL	9.89113	9.86595	9.88	0.02	0.18
		1.1 mL	8.23174	8.24957	8.24	0.01	0.15
IMP-2		0.9 mL	29.33763	28.65601	29.00	0.48	1.66
		1.1 mL	24.36624	24.39978	24.38	0.02	0.10

Ruggedness:

Ruggedness was evaluated to assess the reproducibility of the analytical method under normal but variable conditions, particularly with respect to analyst-to-analyst variation. A standard solution of SITA (6 µg/mL) containing fixed concentrations of the related impurities IMP-1 and IMP-2 was analyzed independently by two different analysts, with each analyst performing two replicate determinations under identical chromatographic conditions. The inclusion of fixed impurity concentrations during ruggedness evaluation was intended to simulate routine analytical conditions encountered during quality control and stability testing, where impurities are expected to be present in the sample matrix.

Ruggedness evaluation:

Peak Name	Parameter change	Peak Area I	Peak Area II	Mean Peak Area	SD	%RSD
SITA	Analyst I	135.5218	137.6374	136.58	1.4096	1.0095
	Analyst II	136.4309	139.7237	138.08	2.3328	1.6886
IMP-1	Analyst I	10.00805	9.90343	9.96	0.0074	0.7443
	Analyst II	9.89287	10.0614	9.98	0.119	1.1994
IMP-2	Analyst I	30.8567	30.14297	30.50	0.505	1.655
	Analyst II	30.5678	31.0175	30.79	0.318	1.033

Conclusion:

A reliable, validated, and stability-indicating RP-HPLC method was successfully developed for the estimation of Sitagliptin and its related impurities (IMP-1 and IMP-2) in tablet dosage forms. The developed method demonstrated excellent specificity, linearity, precision, accuracy, robustness, and ruggedness in accordance with ICH guidelines.

Forced degradation studies confirmed the stability-indicating capability of the method, as degradation products were well separated from the analyte and impurity peaks under all applied stress conditions. The method showed consistent performance in the presence of impurities, making it suitable for impurity profiling, routine quality-control testing, and stability studies.

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