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Original Article

Development of Novel RP-HPLC and Chromogenic UV-Visible Methods for the Estimation of Sitagliptin Using NQS in Bulk and Biological Samples in Compliance with ICH M10 and Q2(R2) Guidelines

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ABSTRACT

Aim: This study develops and validates two analytical methods: An innovative UV-visible chromogenic spectrophotometric method applied to bio-samples and a (RP-HPLC) High-performance liquid chromatography method in reversed phase to measure sitagliptin in pure and pharmaceutical formulations in compliance with M10 and ICH Q2(R2) criteria. **Materials and Methods:** Method utilizes a chromogenic reaction, where 1,2-naphthoquinone-4-sulfonate was prepared and optimized as the chromogenic reagent. This reagent reacted with sitagliptin under controlled pH and temperature to produce a coloured product. The resultant chromogen's absorbance was measured spectrophotometrically at its maximum absorption wavelength. Concentration ranges for RP-HPLC were 25-150 µg/mL. **Results:** Mobile phase for RP-HPLC was made by combining methanol of HPLC grade with potassium dihydrogen phosphate pH 4.3 in 70:30 ratio, 10 min of run time at a flow rate of 1.2 mL/min. Retention time was recorded at 3.41min. Chromogenic method demonstrated a linear response throughout a specific concentration range. calibration curve equation for chromogenic bioanalytical method at 454nm and y=0.0187x+0.0264, R^2=0.9998. 5 µg/mL and 100 µg/mL were found to be the Lower Limit of Quantification (LLOQ) followed by Upper Limit of Quantification (ULOQ), respectively for chromogenic method. **Conclusion:** Both RP-HPLC and chromogenic UV spectrophotometric methods provide rapid as well accurate quantification of sitagliptin in formulations and bulk drug samples. Protein precipitation extraction method was used to design, validate, and expand improved procedure to biological samples. Validation results support their suitability for routine quality control analysis, offering reliable and efficient tools for sitagliptin quantification.

Keywords: ICH M10 Guideline, ICH Q_2R_2, NQS Reagent, RP-HPLC, Sitagliptin, Spectrophotometric Method.

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INTRODUCTION

A medication that is employed to diagnose and treat type 2 diabetes is sitagliptin. IUPAC name 7-[(3R)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl)butyl]-5,6,7,8-tetrahydro-[3-(trifluoromethyl)-1,2,4-triazolo[4,3-a] pyrazine phosphate (1:1) monohydrate. It is member of the group of medications known as Dipeptidyl Peptidase-4 (DPP-4) inhibitions. via promoting absorption of insulin and inhibiting the synthesis of glucagon, sitagliptin lowers blood sugar levels via raising the body's levels of incretin hormones. Sitagliptin can be taken either alone or in combination with other medications that treat diabetes, such insulin, sulfonylureas, or metformin to help persons in type 2 diabetes mellitus improve their glycaemic control. FDA approval year was 2006. Soluble with organic solvents including dimethyl formamide, methanol, and DMSO. Sitagliptin's solubility varies with pH, being more soluble in acidic environments than in neutral or basic ones. Structure of sitagliptin was shown in Figure 1 in mechanism of action of sitagliptin with NQS Reagent. From Literature Review Sitagliptin which shows good water solubility and pH-dependent solubility. Its solubility increases in acidic to neutral pH and decreases in basic media. Several studies have evaluated its solubility in aqueous and non-aqueous solvents, and temperature-dependent solubility behavior has also been reported.

For analytical method development, numerous UV-visible spectrophotometric and RP-HPLC methods have been established

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for assay and dissolution studies in bulk and tablet dosage forms. Typical HPLC conditions employ C18 columns, phosphate buffer with methanol or acetonitrile, and UV detection at 260-270 nm. These methods are validated for linearity, precision, accuracy, and robustness according to ICH guidelines.

Stability-indicating HPLC methods have been developed to separate sitagliptin from its degradation products under stress conditions, while LC-MS/MS methods are used for high-sensitivity quantification in plasma.

ICH Q2(R2) Guidelines

The ICH Q2(R2) guideline (2024) provides updated international standards for the validation of analytical procedures. It outlines key performance characteristics such as accuracy, precision, specificity, linearity, range, detection limit, quantitation limit, robustness, and system suitability. The revision emphasizes a science- and risk-based approach, ensuring analytical methods are reliable, reproducible, and suitable for their intended purpose in pharmaceutical quality control.

Introduction for 1,2-Naphthoquinone-4-sulfonate (NQS) reagent

1,2-Naphthoquinone-4-sulfonate (NQS) is chemical reagent that is often used in analytical chemistry, especially in colorimetric experiments for the detection of certain pharmaceuticals, amino acids, and phenols. Folin first used NQS as a reagent for the calorimetric measurement of amino acids in 1922. He initially presented a technique for identifying amino acids that relies on the formation of brightly colourful compounds by the interaction of amino acid groups using NQS in a solution that is alkaline. 1,2 napthaquinone-4-sulphonate sodium is its chemical name. NQS is fluorescent and will generate an intense red colour with alkaline solutions. The primary and secondary amino acids can react with NQS in basic media and at moderate temperatures to yield spectrophotometrically detectable. Molecular Formula is $C_{10}H_5O_5SNa$ as well Molecular Weight is 276.2 g/mol.[1-4]

Mechanism of action

Sitagliptin has a primary amine that has nucleophilic properties. Electrophilic carbonyl groups found within the quinone ring inside the NQS reagent are particularly susceptible to nucleophilic attack. Primary amine in sitagliptin targets the electrophilic carbonyl carbon of the NQS reagent. This nucleophilic substitution results in the formation of a covalent link between sitagliptin and NQS. This reaction is usually carried out using a basic buffer, which stabilizes the development of the resulting orange-coloured complex and encourages the nucleophilic assault.[5-7] Mechanism of action of sitagliptin with NQS Reagent was shown in Figure 1.

MATERIALS AND METHODS

Chemicals And Reagents

For UV-visible spectrophotometric method, 1,2-Napthoquinone, 4-Sulphonate (Folin's) Reagent, Methanol, 14 pH Buffer solution, Distilled water, Isopropanol, Sitagliptin pure API was a gift sample form the pharmaceutical business and Sitagliptin tablet dosage form was purchased from a nearby pharmacy, samples of plasma were collected from a blood bank. HPLC-grade methanol in order potassium dihydrogen phosphate, and distilled water were used in this procedure.

Instrumentation and analytical conditions

RP-HPLC, ELICO SL-210 double beam UV-visible spectroscopy, and SYSTRONIC SS203. Digital weighing balances were used for sensitively weighing drugs and other powdery materials. Spectra Treats for UV-visible spectrophotometer, LC solutions software, and a Shimadzu LC-20AD system were utilized for the HPLC analysis. A UV detector with a setting of 272 nm was used for the detection. For chromatographic separation, the Phenomenex column of C18 ODS (250 mm × 4.5 mm and 5 µm particle size) was employed. The mobile phase has been made up of methanol of HPLC grade as well potassium dihydrogen phosphate pH 4.3 in 70:30 ratio. Mobile phase had been supplied with a 1.2 mL/min flow rate after being completely degassed before to use.

RP-HPLC method development for sitagliptin analysis

Preparation of standard solution: Pure API sitagliptin (10 mg) was properly weighed before and placed into a volumetric container with a capacity of 10 mL. Stock solution of 1000 µg/mL was made by adding HPLC-grade methanol and make up the volume. Then from 1000 µg/mL prepared 25, 50, 75, 100, 125 as well 150 µg/mL of different concentrations by serially diluting the standard.

Selection of wavelength for sitagliptin

10 ppm sitagliptin was prepared and analyzed using a UV-visible spectrometer in scan mode. The wavelength was estimated by obtaining measurements between 200 and 400 nm. UV absorbance peak was observed at 272 nm.

Optimized method for analysis of sitagliptin

Chromatographic conditions were adjusted for the RP-HPLC procedure to efficiently separate and retain sitagliptin. A C18 column (Phenomenex, 250 mm×4.5 mm, 5 µm) had been utilized for the analysis. HPLC-grade methanol along with potassium dihydrogen phosphate (pH 4.3) were combined in a 70:30 ratio to create mobile phase. The entire process was conducted in isocratic mode for a total of 10 min, employing an injection volume about 20 µL as well as an injection flow rate about 1.2 mL/min. Accurate retention as well peak symmetry were indicated by the chromatogram's Gaussian peak. The efficiency of the column

and the dependability of the procedure were confirmed by the retention time, The theoretical plate count was found to be above 2000, through a retention time period of 3.41 min.

HPLC validation parameters in accordance with Q2R2

Specificity

The specificity can be determined by looking at the interference caused by the improved method. Interference peaks shouldn't show up within blank samples when using these medications. when employing this method. Consequently, it was claimed that this method was distinct. By infusing blank solutions, specificity was achieved.

Systm suitability parameters

Retention time is very consistent (RT %RSD=0.099%) - excellent and well within typical system-suitability expectations. Mean theoretical plates (~3596) indicate acceptable column efficiency (≥2000 typical). Mean tailing (1.075) indicates good peak symmetry (≤2 acceptable) as shown in Table 1a.

Linearity

10 mg of pure API sitagliptin were carefully weighed and dissolved in 10 mL of HPLC-grade methanol in a freshly cleaned volumetric flask to produce 1,000 µg/mL (1000 ppm) of stock concentration. This stock solution was diluted utilizing methanol to provide the serial dilutions of 25, 50, 75, 100, 125, as well 150 µg/mL were prepared as indicated in Figures 2, 3, and Table 1b.

Precision

To create a standard solution of sitagliptin at 50 µg/mL, then 0.5 mL of a 1000 µg/mL solution of standard SGT pipetted into a 10 mL volumetric container and volume has brought on utilizing HPLC-grade methanol. To evaluate the accuracy of the procedure, this solution was made 5 times under identical circumstances. Inter-day accuracy was also evaluated.[8-13]

Accuracy

Recovery tests were conducted at the following levels to evaluate accuracy: 50 along with 100, and 150% of target concentration. At each step, 1 mL of standard solution with the necessary concentration was mixed with 1 mL of 50 µg/mL sitagliptin sample solution. The final amount has been raised to 10 mL by adding diluent. As shown in Table 2, every level has been analyzed in triplicates.

- The sample had been spiked with standard of 25 µg/mL at 50% level.
- Sample had been spiked with standard 50 µg/mL at 100% level.
- A standard containing 75 µg/mL was added to the sample at the 150% level. Each level's percentage recovery was computed in triplicate, demonstrating the accuracy and reliability of the procedure.

LOD and LOQ

Minimum amount of analyte that may been detected is referred to as detection limit. The lowest analytical concentration that can be measured and Quantified is known as the quantitation limit.

Robustness

By analyzing impact of minute changes in flow rate on reference solution's peak regions, robustness was evaluated. Two distinct flow rates 1.15 and 1.25 mL/min-were used for the analysis, which somewhat deviated from the recommended flow rate. Six replicate injections were carried out at each flow rate, and the associated peak areas were noted. Additionally, changes in the UV detector's λ_{max} were noted in the HPLC parameters. To assess the method's dependability in these altered circumstances, the %RSD was calculated as shown in Table 3.

Assay

The brand of sitagliptin tablets utilized was sitadiet, which was purchased from a local pharmacy. 1000 µg/mL solution of stock was made by grinding ten 100 mg tablets into very fine powder, and 10 mg of powdered form taken in 10 mL volumetric flask 0.5 mL was taken from 1000 ppm to make a 50 µg/mL working solution. A chromatographic method was used to analyze the sample, and the peak area was measured. The potency of the sitadiet tablets was determined by substituting the active ingredient's % assay by entering the resulting peak area within a calibration calculation.

Chromogenic method by UV-visible spectrophotometer

Standard stock solution (1000 ppm)

Weighed 0.01 g of Sitagliptin pure drug in 10 mL volume flask, dissolved and added upto mark 10 mL with methanol. Resultant standard stock solution was found to be 1000 µg/mL.

Buffer solution of pH 14

4 g of Sodium Hydroxide (salt of strong base) was taken and dissolve 2 g of Potassium Chloride (salt of strong acid) in 100 mL Volumetric flask with distilled water.

Preparation of 1,2-Napthoquinone 4-Sulphonate Reagent

Taken 0.05 g of NQS reagent in 50 mL volume flask, then remaining portion was filled with distilled water.

Order for addition of reagents: 1 mL of standard solution (SGT) was taken to that 1 mL of 14 pH Buffer solution was added next to that added 1 mL of 1,2-Napthoquinone, 4- Sulphonate was added formation of reddish orange coloured complex. The optimized

Table 1a: System Suitability Parameters for Sitagliptin.

Parameter	Acceptable Criteria	Typical Observation
Retention Time	Within expected range	Generally observed between 3-6 min, depending on chromatographic conditions.
Theoretical Plates (N)	≥ 2000	Usually found above 3000-4000 indicating good column efficiency.
Tailing Factor (T)	≤ 2.0	With in limits confirming symmetrical peak shape.
Resolution	≥ 2.0	Ensures adequate separation from other components or degradation products.
%RSD of Peak Area (Repeatability)	≤ 2.0%	<1.0%, showing high injection precision.
Peak Asymmetry	≤ 1.5	Reflects uniform and sharp peak profile.
Signal-to-Noise Ratio	≥ 10 at LOQ	Confirms adequate system sensitivity for quantification.

Table 1b: HPLC linearity data.

Concentration µg/mL	Area	Retention Time	Theoretical Plate	Tailing Factor
25 µg/mL	686788	3.412	3678.3	1.06
50 µg/mL	795316	3.415	3934.2	1.01
75 µg/mL	899754	3.411	3392.6	1.12
100 µg/mL	989984	3.417	3567.4	1.14
125 µg/mL	1098768	3.411	3876.6	1.05
150 µg/mL	1189865	3.419	3128.5	1.07

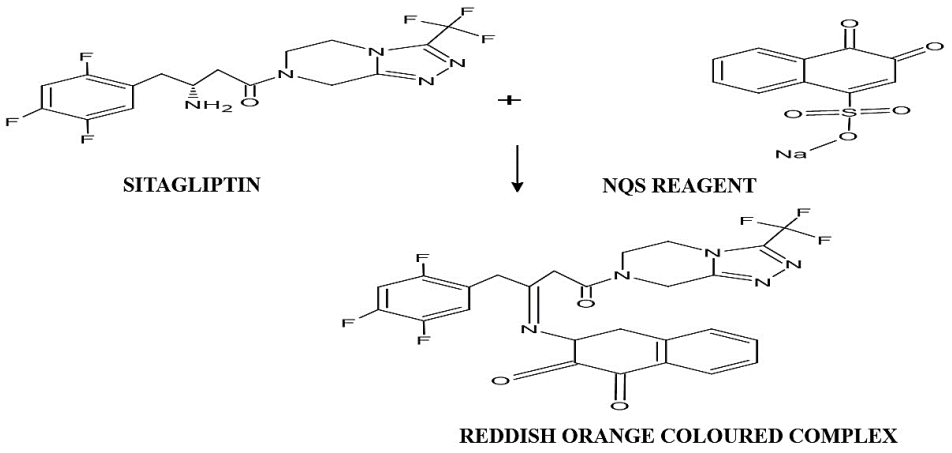

Figure 1: Reaction of sitagliptin with NQS Reagent.

wavelength is 454 nm. The optimized temperature is 24°C for 1 hr, the colour is optimized and is reddish orange colour.

Colour stability: The drug has a 2-hr colour stability period. Due to extended temperature changes and temporal fluctuations, colour has faded after 2 hr.

Chromogenic Bio-Analytical Method by UV-visible Spectrophotometer

Plasma preparation: Purchased from blood bank.

Preparation of standard solution (10 µg/mL): After pipetting 0.1 mL of working standard solution from 1000 ppm into a 10 mL volume flask methanol had been utilized in order to reach the mark.

Preparation of blank plasma: Reagents were added after 1 mL of plasma had been measured and transferred to 10 mL volumetric flask including methanol.

Extraction steps for chromogenic bioanalytical method

A bioanalytical method was performed using protein precipitation. Plasma samples were stored in refrigerator and then thawed at room temperature on the day of analysis.[14-17] To 1

mL of plasma sample 0.5 mL of 50 ppm sitagliptin standard drug from 1000 µg/mL was added, to it 2 mL of diethyl ether and 4 mL of isopropanol had been added, entire mixture was centrifuged in 1000 rpm for 50 min. Liquid supernatant was poured into 10 mL volumetric flask. 1 mL of NaOH and 1 mL of NQS reagent are added to 10 mL vol flask a diluent is used to bring the final volume to 10 mL absorbance of the solution is analysed at 454 nm using corresponding reagent blank. % recovery of bioanalytical method was found to be 98.58% and spectrum was shown in Figure 4.

RESULTS

Chromatographic Method

Linearity

For 25-150 µg/mL the linearity of sitagliptin is Y=4018.3X + 591815, R^2=0.9992.

Precision

Intraday and Interday Precision

The sitagliptin's %RSD of repeatability was 0.01005455%. In the morning, the intra-day precision %RSD was 0.01005455%, and on Inter day the first and second days, the values were 0.013541343% and 0.061493662%, In contrast, the evening value was 0.012129139%. Every result met the ICH requirements for being within limits. Effective method reliability and consistency are shown by these outcomes.

Detection and Quantification Limits

Utilizing formulas derived from calibration curve's slope and the standard deviation of regression lines' y-intercept, LOD as well LOQ calculated and had been found to be 0.0656660 µg/mL and 0.1989880 µg/mL.

Robustness

Robustness analysis was conducted utilizing these two wavelengths that are -1(%RSD 0.027044149) and +1(%RSD

Table 2: Accuracy data percentage of sitagliptin recovered.

Percentage Level	Sample 50 µg/mL Area	Standard 25 µg/mL, 50 µg/mL, 75 µg/mL Area	Total Area	% Recovery	Mean % Recovery
50% (50 µg/mL +25 µg/mL)	789998	686788	1476654	99.98%	99.96%
			1476536	99.96%	
			1476548	99.96%	
100% (50 µg/mL +50 µg/mL)	789998	795316	1585121	99.97%	99.96%
			1585101	99.97%	
			1585026	99.96%	
150% (50 µg/mL + 75 µg/mL)	789998	899754	1689624	99.98%	99.97%
			1689543	99.97%	
			1689532	99.97%	

Table 3: Robustness data of HPLC.

Robustness	Flow rate at 1.15 mL/min	Flow rate at 1.2 mL/min	Flow rate at 1.25 mL/min
Concentration	Area	Area	Area
50 µg/mL	794327	795316	796584
50 µg/mL	794339	795326	796538
50 µg/mL	794329	795321	796432
50 µg/mL	794289	795226	796498
50 µg/mL	794154	795126	796686
50 µg/mL	794786	795218	796618
Mean	794370.6667	795255.5	796559.3333
S D	214.8307861	79.95936468	90.02814375
%RSD	0.027044149	0.01005455	0.011302127

Table 4: Matrix effect, With in run precision, Reinjection reproducibility data at 454 nm.

Matrix effect of 15, 75 µg/mL Sitagliptin				
QC Concentration Levels	**Matrices**	**Mean**	**S D**	**%CV**
LQC 15 µg/mL	Matrix 1	0.2578	0.035143207	13.63196549%
	Matrix 2	0.2718	0.030900566	11.36886179%
	Matrix 3	0.269466667	0.037264527	13.828993%
	Matrix 4	0.276933333	0.033304729	12.02626242%
	Matrix 5	0.251333333	0.025667976	10.2127226%
	Matrix 6	0.2570	0.036062446	14.03208009%
HQC 75 µg/mL	Matrix 1	1.4628	0.214936921	14.69352755%
	Matrix 2	1.486466667	0.164566137	11.07096044%
	Matrix 3	1.4928	0.165791677	11.10608765%
	Matrix 4	1.492066667	0.160230376	10.73882149%
	Matrix 5	1.441133333	0.195768264	13.58432699%
	Matrix 6	1.6428	0.168195957	10.2383709%
With in run precision of concentrations 5, 15, 50, 75 µg/mL				
QC Levels	**Concentration**	**Average**	**S D**	**%CV**
LLOQ	5 µg/mL	0.16156	0.031426629	19.45198%
LQC	15 µg/mL	0.25574	0.028373103	11.09451%
MQC	50 µg/mL	0.8495	0.090257476	10.62452%
HQC	75 µg/mL	1.42972	0.163895857	11.46349%
Reinjection reproducibility of QC samples				
LQC	15 µg/mL	0.25534	0.028611589	11.20529042%
MQC	50 µg/mL	0.84552	0.091239312	10.79091112%
HQC	75 µg/mL	1.46532	0.15881175	10.83802515%

0.011302127). Results fell within appropriate limits and meeting ICH requirements.

Assay

$$\% \text{ Assay} = \frac{\text{Sample Area}}{\text{Standard Area}} \times \frac{\text{Standard Concentration}}{\text{Sample Concentration}} \times 100$$

$$\% \text{ Assay} = 1 + \frac{789998}{795316} \times \frac{50}{50} \times 100 = \% \text{ Assay} = 99.33\%$$

$$= 99.33\%$$

Validation parameters for Chromogenic Bio-Analytical Method as per ICH M10 Guidelines

In compliance with ICH M10 guidelines, the bio-analytical method was validated. The following are included in a complete validation:

Specificity and Selectivity, Range, Accuracy & Precision, Matrix Effect, Calibration curve, Reinjection Reproducibility, Stability.[18-20]

Range for Chromogenic bioanalytical method

LL0Q=5 µg/mL

ULOQ=100 µg/mL

Matrix Effect

The matrix effect is the alteration of the analyte response caused by interfering matrix components. Three replications with Low and High Quality Control (QCs) were made using a matrix from six distinct sources/lots, and the matrix effect was then examined. Matrix effect data at 454 nm was shown in Table 4.

Procedure

Preparation of Low QC sample (15 µg/mL) for chromogenic bioanalytical method

1.5 mL of the usual STG solution along with 1 mL human plasma were then pipetted directly into a 10 mL volume flask the drug solution was obtained following centrifugation. Following the collection of the supernatant layer, three replications were made and reagents were added and analyzed. Methanol was added to

the solution and this solution at 463 nm was analyzed over a reagent blank employing UV-visible spectroscopy.

Preparation of Middle QC (50 µg/mL) and High QC (75 µg/mL) for chromogenic bioanalytical method

After centrifugation, 0.5 mL and 0.75 mL of standard drug solution has been pipetted out into separate 10 mL measuring flasks, and 1 mL plasma were pipetted into it. After collecting the supernatant layer, three replicates were made and reagents were added. Methanol was added to the solution to make it up to mark. UV-visible spectroscopy was used to scan the resulting solution at 454 nm against a corresponding reagent blank.

Reinjection Reproducibility

To verify the vitality of treated samples and to promote their storage prior to reinjection, re-injection reproducibility must be evaluated if samples might be reinjected. The procedure's repeatability is assessed through replicated evaluations of the QCs, usually being a component to the accuracy as well as precision assessment.[21] To determine the durability of these treated specimens as well as support their storage before reinjection, reinjection consistency shall be assessed in cases in which samples may be reinjected. Table 4 shows Reinjection reproducibility data at 454 nm.

Procedure

Procedure was performed using Shimadzu 1900i. Reinjecting a run that contains a standard of calibration then a minimum of five injections with middle, low as well high QCs after storage is how reinjection repeatability is assessed.

Calibration curve

Calibration curve shows connection between actual concentration of an analyte and how testing platform responds to it. The calibration standards, that are produced by mixing a specific amount in analytical or analytes into a matrix, make up the calibration curve. Furthermore, it is necessary to employ the same biological matrices that was taken advantage for developing of study's samples for calibration standards.[22-25] Calibration range is defined by LLOQ, as well ULOQ. Every analyte that is examined throughout technique validation and every analytical run ought to have a calibration curve. Figure 5 shows calibration plot at 454 nm.

Procedure for linearity of chromogenic bioanalytical method.

Portions of Sitagliptin standard drug solution in concentration ranges were put into an amount of 10 mL volumetric flasks: 0.05, 1.5, 0.5, 0.75, and 1 mL from 1000 µg/mL was taken. 1 mL plasma spike was added to each 10 mL vol flask. Then added 1 mL of 14 pH buffer solution and 1 mL with 1,2 Naptho quinone 4-sulphonate methanol. Orange coloured chromogen of Sitagliptin standard solutions absorbance at 454 nm was compared against corresponding reagent blank. For each of these variables, the median±standard deviation of the slope, intercept, as well as correlation coefficient was calculated. Figure 5 and Table 5 shows linearity plot for chromogenic bioanalytical method.

Accuracy and precision

The same runs and data should be used to evaluate accuracy as well precision. Exclude the LLOQ, since the nominal concentration should be within ±20% and the accuracy should be within ±15% at each concentration level. At any given level, the accuracy (%CV) of a concentration cannot exceed 15%. with the exception of the LLOQ, where it shouldn't exceed 20%. As both precision as well as non-accuracy validation runs, at least 50% of every concentration level and three-thirds of all QCs must be between ±15% of the nominal values.

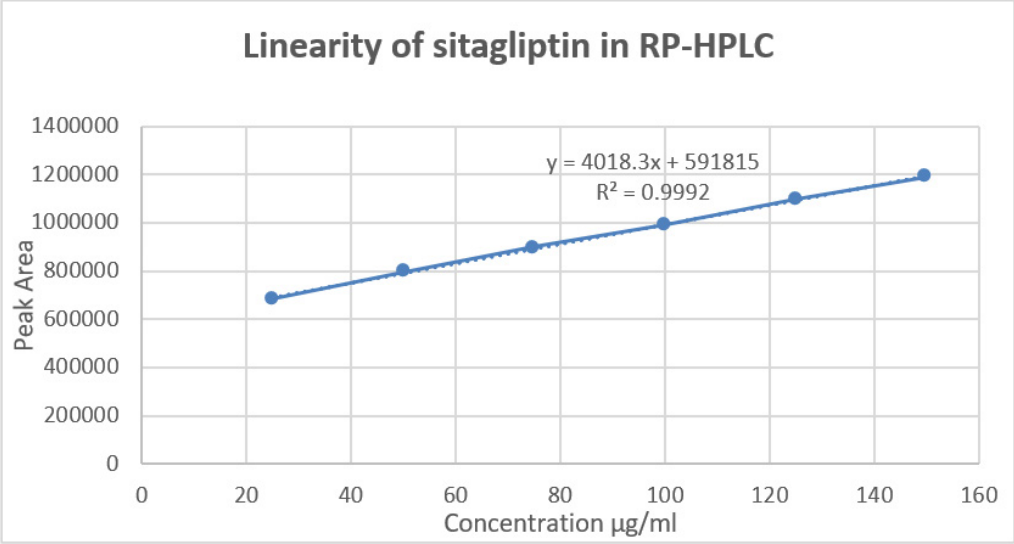

Figure 2: RP- HPLC Linearity of Sitagliptin standard solution.

Table 5: **Between run precision and Bench top stability studies of concentrations at 454 nm.**

Between run precision of concentrations at 454 nm							
QC Levels	Concentration (µg/mL)	Average		S D		%CV	
		Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
LLOQ	5 µg/mL	0.165433333	0.1324	0.02853600	0.0241737	17.2492%	18.2581%
LQC	15 µg/mL	0.251133333	0.240666667	0.02585079	0.02433153	10.2936%	10.11005%
MQC	50 µg/mL	0.866866667	0.85713333	0.09743887	0.09680	11.2403%	11.29404%
HQC	75 µg/mL	1.547133333	1.48086666	0.1824023	0.1795171	11.7896%	12.12243%
Bench top stability studies of LQC 15 µg/mL and HQC 75 µg/mL at 454 nm							
QC Concentration Levels	**Freeze thaw cycles**	**Mean**		**SD**		**%CV**	
LQC 15 µg/mL	1st Freeze thaw cycle	0.251133333		0.02585079		10.29365137%	
	2nd Freeze thaw cycle	0.445866667		0.052962298		11.87850582%	
	3rd Freeze thaw cycle	0.148833333		0.016430561		11.0395706%	
HQC 75 µg/mL	1st Freeze thaw cycle	1.560466667		0.186693903		11.963978%	
	2nd Freeze thaw cycle	1.5176		0.186780727		12.30763884%	
	3rd Freeze thaw cycle	1.460133333		0.191225556		13.09644481%	

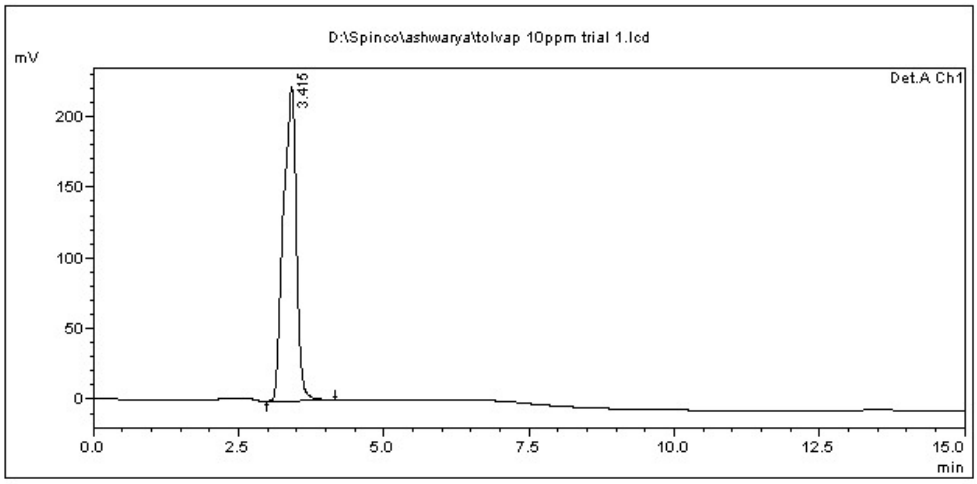

Figure 3: Chromatogram of standard sitagliptin 50 µg/mL.

Procedure for preparation of within run and between run Accuracy and Precision samples

The following ranges of small portions of standard drug solution were placed in various quantities in 10-mL volumetric flasks 0.5, 1.5, 5.0 along with 7.5 mL from 100 µg/mL was taken and 1 mL plasma spike was added to each 10 mL capacity volume flask. After centrifugation, medication solution has been obtained. After collecting the supernatant layer, reagents were added. Methanol was added to the solution to make it up to mark. Their LLQC, LQC, MQC, as well HQC were analysed. The resultant solution has been scanned at 454 nm using UV-visible spectroscopy against reagent blank. For five QC samples, the average±standard deviation and %CV has been determined and all the results were found to be in limits. Tables 4 and 5 shows with in run and between run precisions data at 454 nm.

Stability

To make that the analyte concentration is unaffected by the procedures applied to sample preparation, processing, and evaluation as well as by the storage conditions employed, stability studies are crucial. The evaluation's circumstances must coincide with those of the study samples. The matrix of particles must be spiked for each dosed chemical in order to perform benchtop, freeze-thaw, with long-term stability tests for pharmaceuticals with fixed dose combinations and carefully labelled dosing

regimens.[26-28] Chemical treatments may be expanded to lower temperatures if stability at a certain temperature is demonstrated.

Bench top stability studies (Short-Term)

It is essential to plan and carry out benchtop matrix stability studies that consider the handling circumstances of the study materials in the lab. Thawing high and low QCs on the tabletop should be done in the same temperature and for the same length of time as the study materials.[29] Three freeze thaw cycles were conducted for LQC and HQC samples. Table 5 shows Bench top stability studies data at 454 nm.

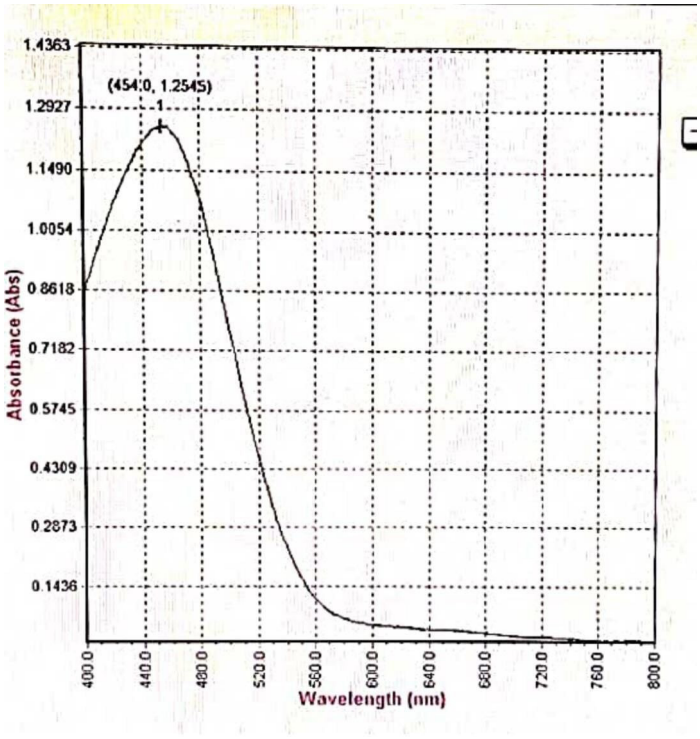

Figure 4: Sitagliptin spectrum of chromogenic bioanalytical method.

DISCUSSION

By utilizing RP-HPLC with UV detection, first method showed accurate and effective sitagliptin separation with 3.41 min of retention time and a distinctive Gaussian peak. Isocratic mobile phase is utilized made up with methanol along with potassium dihydrogen phosphate pH 4.3 (70:30, v/v) with a 1.2 mL/min flow rate separation was accomplished. Detection has been achieved at 272 nm. The correlation coefficient (R^2) was 0.9992, and technique demonstrated excellent linearity over range of levels 25-150 µg/mL. Findings demonstrated a high level of sensitivity, with the Limits of Quantification (LOQ) and Detection (LOD) being 0.1989880 µg/mL as well 0.0656660 µg/mL, respectively. In Compliance with ICH Q2(R^2) requirements, the method's recovery rate of 99.97% was confirmed, proving its accuracy, precision, robustness, as well reproducibility for routine pharmaceutical analysis. Second method examined sitagliptin levels in human plasma samples. An excellent recovery rate of 98.58% was obtained by optimizing a protein precipitation process for the extraction of samples. Sitagliptin in human plasma has been measured using validated chromogenic and UV techniques in accordance with ICH M10 guidance. Tests for matrix effect, linearity of the calibration curve, accuracy, precision, repeatability of reinjection, and stability of the method validation were performed with R^2>0.999, linearity was seen between 5-100 µg/mL at 454nm. LQC, MQC, and HQC samples remained below acceptable limits (<15%) and extraction recoveries were appropriate, while precision values %CV for LLOQ were within 15-20%.

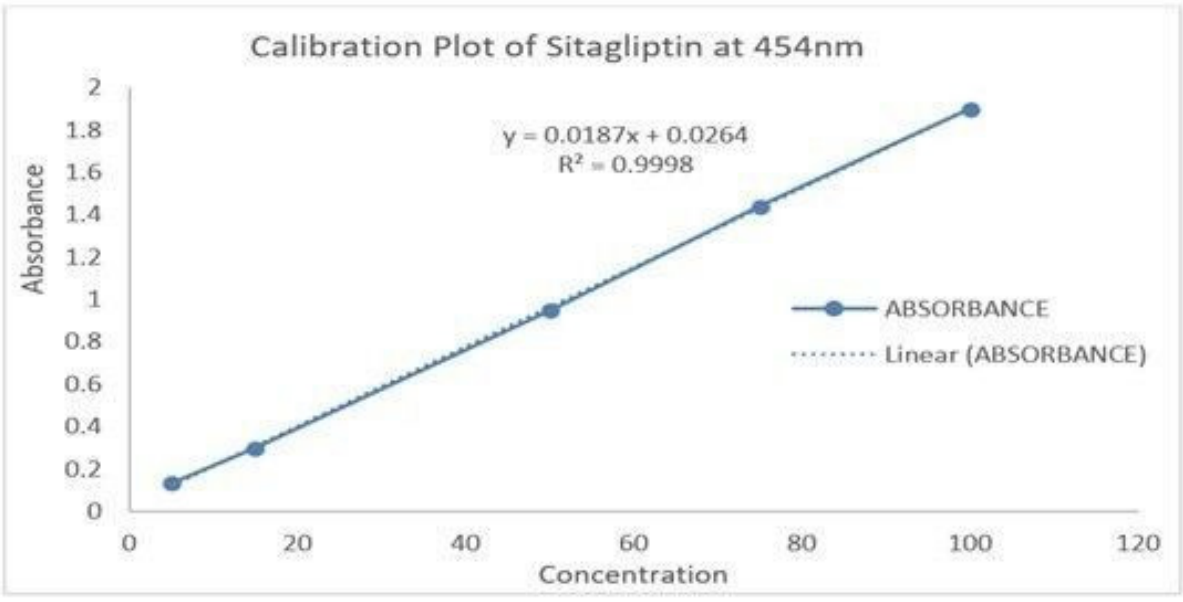

Figure 5: Linearity plot for chromogenic bio-analytical method.

CONCLUSION

RP-HPLC-UV method offers rapid, simple and extremely accurate process with minimal solvent usage and consistency with labeled claims. Efficient analytical technique for sitagliptin quantification by 1,2-naphthoquinone-4-sulphonate (NQS) was accurate. Under ideal circumstances, sitagliptin and NQS reacts to generate stable, highly coloured complex that spectrophotometry can detect. Because the reaction with NQS creates a distinct chromophore that can be detected at a particular wavelength, the approach is specific to sitagliptin and reduces interfering from excipients into pharmaceutical formulations. Within a given concentration range, the method exhibits strong linearity, guaranteeing precise sitagliptin measurement. The method is sensitive enough to trace-level determination because detection limit LOD as well quantification LOQ are appropriate for regular analysis. These methods demonstrated excellent linearity, accuracy as well precision, fulfilling the prerequisites for routine bioanalytical applications.

The simplicity and methods' cost-effectiveness make them particularly advantageous in clinical settings. Successful application of these methods to human plasma samples suggests their potential for the pharmacokinetic analysis of sitagliptin and therapeutic medication monitoring. These newly developed spectrophotometric methods offer a reliable and efficient approach in order to measure sitagliptin levels in human plasma samples, suitable for routine analysis in clinical and research laboratories.

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ABBREVIATIONS

SD: Standard deviation; **UV:** Ultraviolet; **% RSD:** Relative standard deviation; **SGT:** Sitagliptin; **LLOQ:** Lower limit of quantification; **LOQ:** Limit of quantitation; **CV:** Coefficient of variance; **LOD:** Limit of detection; **ICH:** International Council on Harmonization; **PPM:** Parts per million; **RP:** Reverse phase; **µg:** Microgram; **ULOQ:** Upper limit of quantification; **mg:** Milligram; **mL:** Milliliters; **%:** Percentage; **HPLC:** High-performance liquid chromatography.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

The human plasma used in this study was obtained from blood banks.

SUMMARY

The study details the development and validation of two analytical methods for estimating sitagliptin: an RP-HPLC method and an innovative chromogenic UV-visible spectrophotometric method Utilizing 1,2-Naphthoquinone-4-Sulfonate (NQS). Both methods quantify sitagliptin in bulk drug, pharmaceutical formulations, and biological samples (human plasma), complying with ICH M10 and Q2(R2) guidelines.

The RP-HPLC method used a C18 column and an isocratic mobile phase of methanol and potassium dihydrogen phosphate (70:30, pH 4.3), achieving detection at 272 nm with a retention time of 3.41 min. It showed excellent linearity over 25-150 µg/mL (R^2=0.9992) and a high recovery rate of 99.97%. The chromogenic UV method uses NQS, which reacts with sitagliptin's primary amine to produce a reddish-orange complex detected at 454 nm. Optimized for bio-samples via protein precipitation extraction, this method was linear from 5-100 µg/mL and demonstrated a high recovery rate of 98.58% in human plasma. These rapid, accurate, and cost-effective methods are suitable for routine quality control and pharmacokinetic analysis.

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