Validation Of Sitagliptin Phosphate & Simvastatin By RP-HPLC

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ABSTRACT

The importance of antidiabetic drugs are increasing day by day generally Sitagliptin phosphate is a highly selective DPP-4 inhibitor that is thought to act in type 2 diabetes by slowing the inactivation of incretin hormones and Simvastatin increases the rate at which the body removes cholesterol from blood by HMG-CoA reductase inhibition. So, both of these drug in combinationare used for method development by RP- HPLC. Few analytical techniques are reported for STG and SMV out of which many of these are time consuming, affecting column life and costly. So this research work is dedicated to develop and validate simple, efficient, economical, fast, reliable new method for Simultaneous estimation of STG and SMV in bulk and pharmaceutical dosage form by RP- HPLC. In this method analytical technique of Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was used for quantitative determination of Sitagliptin Phophate & Simvastatin was used. The mobile phase used was Methanol : Water (90:10).

Keyword : - *Sitagliptin Phosphate1, Simvastatin2, Mobile Phase3, etc….*

1. Introduction

Analytical Chemistry is a scientific discipline that develops methods, instruments & strategies to obtain information on the composition & nature of matter. Analytical chemistry is concerned with the chemical characterization of matter & thus pharmaceutical analysis covers matter having pharmaceutical applications. Knowledge of chemical composition of many substances is important in daily life. Analytical chemistry plays an important role in nearly all aspects of chemistry viz. agriculture, clinical, environmental, forensic, manufacturing, metallurgical & pharmaceutical chemistry. At present several analytical methods are available for analyzing analyte viz. spectroscopic & chromatographic. Spectroscopic method includes UV-visible, infrared, mass, NMR, absorbance spectroscopy while chromatographic methods include high performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), super-critical chromatography, gel permeation chromatography methods etc.[1]

1.1 Chromatography

Chromatography is defined as a procedure by which solutes are separated by a dynamic differential migration process in a system consisting of two or more phases, one of which moves continuously in a given direction and in which the individual substances exhibit mobilities by reason of differences in adsorption, partition, solubility, vapour pressure, molecular size or ionic charge density. The individual substances thus obtained can be identified or determined by analytical methods. Chromatography was first invented by Michael Tswett, a Russian botanist in 1906 for the separation of colored substance into individual component. Chromatography was invented nearly 100 years ago, but it is only in the past few years that the development of the technique and associated instrumentation has reached a level that might be called the steady state.6,7

1.2 Chromatographic Parameters

Table 1 Characteristics to be validated in HPLC

Characteristics	Acceptance Criteria
Accuracy	Recovery 98-102%
Precision	RSD<2%
Repeatability	RSD<2%
Specificity	No interference
Detection Limit	S/N>2 or 3
Quantitation Limit	S/N>10
Linearity	Correlation coefficient r>0.999
Range	80-120%

2. Material & Methods

Table 2 Chemical & Reagent

Sr. No.	Chemical / Reagent / Solvent	Grade	Make	Batch No.
1	Methanol	HPLC	Merck lab.	S3SA31599
2	Water	HPLC	Mill-Q/Merck Lab	NA

Table 3 Instruments

Sr. No.	Instrument	Make	Model	Calibration Date
1	UV	Shimadzu	1800	30 April 2023
2	HPLC	Shimadzu	LC2030-3D	15 Nov 2023

2.1 Raw Material Characterization

2.1.1 Characterization of Sitagliptin Phosphate (STG)

Determination of Melting Point
Melting point was determined using digital malting point apparatus. The reference melting point of Sitagliptin Phosphate is 204^0C.

Determination of λ max UV
1000µg/ml solution of STG was prepared by accurately weighing 100mg of STG. It was then transferred to 100ml volumetric flask containing solvent mixture. Finally the volume was made up to the mark using solvent mixture. The solution was scanned by using UV visible spectrophotometer in the range of 200-400nm. The reference λmax of STG is 268 nm (Shimadzu 1800).

2.1.2 Characterization of Simvastatin (STV)

Determination of Melting Point
Melting point was determined using digital malting point apparatus. The reference melting point of Simvastatin is 129^0C.

Determination of λ max UV
1000µg/ml solution of STV was prepared by accurately weighing 10mg of STV. It was then transferred to 100ml volumetric flask containing solvent mixture. Finally the volume was made up to the mark using solvent mixture. The solution was scanned by using UV visible spectrophotometer in the range of 200-400nm. The reference λmax of STV is 237 nm (Shimadzu 1800).

2.2 Experimental Work

2.2.1 UV Method Development

2.2.1.1 Preparation of Stock Solution

Standard Stock Solution of Sitagliptin Phosphate (STG)

100 mg of STG was weighed accurately and transferred to a 100 ml volumetric flask containing some amount of solvent mixture. Volume was made up to the mark using solvent mixture to obtain the final stock solution of 1000 µg/ml. UV visible spectrophotometer in range 200-400nm The absorbance of this solutions was recorded by using UV spectrophotometer (Shimadzu 1800).

Standard Solution of Simvastatin (STV)

10 mg of SMV was weighed accurately and transferred to a 100 ml volumetric flask containing some amount of solvent mixture. Volume was made up to the mark using solvent mixture to obtain final stock solution of 100 µg/ml. The absorbance of the latter was recorded using UV visible spectrophotometer in range 200-400nm. The absorbance of this solution was recorded by using UV spectrophotometer (Shimadzu 1800).

Standard Stock Solution of Sitagliptin Phosphate (STG) & Simvastatin (SMV) Mixture

Weigh an accurate quantity of Sitagliptin phosphate (STG) and Simvastatin (SMV) 100 mg and 10 mg respectively was transferred to the 100 ml volumetric flask and dissolved in solvent mixture. Finally the volume was made up to the mark with solvent mixture to obtained the consequential concentration 1000 µg/ml STG and 100 µg/ml SMV respectively. The absorbance of mix solution was recorded by using UV spectrophotometer (Shimadzu 1800).

Preparation of working solution

6.0 ml was pipette out and diluted up to 10 ml which will give resultant solutions of 600 µg/ml STG and 60 µg/ml SMV respectively. Six replicates of the solution were performed and absorbance was recorded at 252nm. Mean, SD and %RSD was calculated.

2.2.1.2 Method Validation

Linearity

From stock solution 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml solutions were pipetted out and diluted up to 10 ml using solvent mixture to obtain resultant solutions of 100-600 µg/ml STG and 10-60 µg/ml SMV. Absorbance for each of these solutions were recorded in triplicate and calibration curve was constructed considering mean absorbance of each test solution. From the calibration curve equation of line, correlation coefficient and intercept was determined.

Precision

From the calibration range three QC standards was defined viz. 150, 350 and 550 µg/ml of STG and 15, 35 and 55 µg/ml of SMV as LQC, MQC and NQC respectively. The solutions for QC standards was prepared by diluting stock solution of 1.5, 3.5 and 5.5 ml solutions up to 10ml. Absorbance of each QC standard was recorded for intra day and inter day precision in triplicates as per ICH guidelines Q2R1.

Accuracy

% Accuracy was determined using observations of precision study using following formula.

%Accuracy = Mean measured conc. – Nominal / Nominal * 100

Robustness

10µg/ml solution was selected for robustness study for the parameters like wavelength. Wavelength was subjected to minor variation of ±1 (viz.252±1). The absorbance for each of these wavelengths was recorded in triplicate. The variation should not be more than 5% RSD.

LOD & LOQ

LOD and LOQ were determined using following formulas

LOD = 3.3 * S.D / S

LOQ = 10 * S.D / S

2.2.2 HPLC Method Development

From results obtained from UV method methanol and water (90:10) was selected as mobile phase for HPLC method development.

2.2.2.1 Preparation of Stock Solutions

Preparation of working solution of STG & SMV in mixture

Accurately weighed 100 mg of Sitagliptin phosphate and 10 mg of Simvastatin, transferred to 100 ml volumetric flask containing a mixture of methanol and water in the ratio of 90:10. The volume was made up to the mark using same mixture of mobile phase. The resulting solution was filtered through 0.45µ membrane and sonicated for three cycles each of 10 min.

System Suitability Testing

Preparation of working solution

4 ml stock solution was pipetted out and diluted up to 10 ml to obtain resultant solution of 400 µg/ml and 40 µg/ml STG & SMV respectively. Seven replicates of this solution were injected and results were recorded for RT, peak area, tailing factor (symmetry factor) and theoretical plates. Mean, SD and %RSD were calculated for the results obtained as well as other parameters were also verified for acceptability level.

1) The column efficiency for STG and SMV should not less than 2000 theoretical plates.
2) The tailing factor for peak, should not more than 2.0.
3) % CV for area shall NMT 1.5 and for RT NMT 0.5%

2.2.2.2 Method Validation

Linearity

From stock solution 4-10ml was pipetted out and diluted up to 10 ml to obtain 400-1000µg/ml and 40-60µg/ml resultant solutions of STG and SMV respectively. The resulting solution was filtered through 0.45µ membrane and sonicated for three cycles each of 10 min. Calibration curve was constructed between concentration Vs peak area. Results were recorded for equation of line, correlation coefficient and intercept were determined.

$$Y = mX + c$$

Precision

From the calibration range three QC standards was defined viz. 450, 650 and 950 µg/ml of STG and 45,65 and 95 of SMV as LQC, MQC and NQC respectively. The solutions for QC standards were prepared by diluting stock solution of 4.5, 6.5, and 9.5ml solutions up to 10 ml. Area of each QC standard were recorded for intra day and inter day precision in seven replicates as per ICH guidelines Q2R1 . Results were recorded to calculate mean, SD, %RSD etc.

% Accuracy

% Accuracy was determined using observations of precision study using following formula.

$$\%Accuracy = Mean\ measured\ conc. - Nominal / Nominal * 100$$

Robustness

500 µg/ml solution was selected for robustness study for the parameters like mobile phase proportion, flow rate etc. Seven replicates for parameters given in table were injected and area for each of the parameter was recorded. The variation should not be more than 5% RSD. One factor was changed at time to estimate the effect.

Table 4 Robustness Variation Table

Condition	Normal	Variation 1	Variation 2
Mobile Phase	90:10	91:09	89:11
Flow Rate	1 ml/min	1.05 ml/min	0.95 ml/min

LOD & LOQ

LOD and LOQ were determined using following formulas

$$LOD = 3.3 * S.D / S$$
$$LOQ = 10 * S.D / S$$

% Recovery

Preparation of Stock Solution
Accurately weighed 100 mg of STG (API) and 10 mg of SMV (API) was added in volumetric flask containing some amount of mobile phase and volume was made up to the mark using mobile phase. The resulting solution was filtered through 0.45µ membrane filter and sonicated for three cycles each of 10 min. From the stock solution 5.0ml of stock was pipette out in triplicate and kept in three different volumetric flasks, cleaned previously and diluted up to 10ml by using mobile phase to obtain resultant solution of 500µg/ml and 50µg/ml. This solution was injected for given chromatographic system in triplicate and mean area was determined.

Preparation of stock from dosage form
Twenty tablets (Label claim 110 mg of STG and SMV, JUVISYNC, Merk Ltd.) were weighed, average weight was determined and powdered. Powder equivalent to 100 mg STG (227 mg) and 10 mg (227 mg) was transferred to 100 ml of Mobile phase. The resulting solution was filtered through 0.45µ membrane filter and sonicated for three cycles each of 10 min. From the stock 4.8, 5.0, 5.2 ml solutions were pipetted out and diluted up to 10 ml using mobile phase to obtain resultant solution of 480, 500 and 520 µg/ml.

Preparation of test solution for % recovery by spike method
500 µg/ml solution of (API) was spiked into each of above dilutions of 480, 500 and 520 µg/ml to obtain solutions at 80%, 100% and 120% respectively. Each of these three levels were injected in triplicate and mean area for each level was determined. The mean area obtained on API injection was subtracted from the mean area of each of these three levels to obtain area corresponding to test solutions. % recovery was determined from the test and standard area using following formula.

% Recovery = A – B/C * 100

2.2.3 Standard Testing Procedure
2.2.3.1 Chromatographic Parameters

Table 5 Chromatographic Parameters

Column	Phenomenex C18 (4.6*250 mm)
Particle Size	5 µm
Column Temperature	Ambient
Autosampler Temperature	Ambient
Detector Wavelength	UV@253
Flow	Isocratic
Flow Rate	1 ml/min
Injection volume	10 µl
Needle Wash	Methanol : Water (90:10)
Diluent	Methanol : Water (90:10)
Mobile Phase	Methanol : Water (90:10)

2.2.3.2 Preparation of Standard & Sample
Preparation of Standard Stock Solution
Accurately weighed 100 mg of Sitagliptin phosphate standard and 10 mg of Simvastatin standard, transferred to 100 ml volumetric flask & diluted upto mark with diluent. The resulting solution was filtered through 0.45µ membrane and sonicated for three cycles each of 10 min.

Preparation of Sample
Accurately weighed 100 mg of Sitagliptin phosphate sample and 10 mg of Simvastatin sample, transferred to 100 ml volumetric flask & diluted upto mark with diluent. The resulting solution was filtered through 0.45µ membrane and sonicated for three cycles each of 10 min.

System Suitability Solution
Transfer 4 ml of Standard stock solution in 10 ml Volumetric flask & dilute upto mark with diluent. The resulting solution was filtered through 0.45µ membrane and sonicated for three cycles each of 10 min.

Sequence

Table 6 Sequence

Solution	Injection
Blank	2
System Suitability Solution	1
Standard Injection	5
Sample Injection	2
Bracketing Standard Injection	1

System Suitability Parameter

- Blank is free from interference at RT of Sitagliptin Phosphate & Simvastatin.
- RSD of Area & RT (5 Std. Injection) shall be less than or equal to 2.
- Theoretical plates shall be greater than 2000.
- S/N ratio shall be greater than or equal to 10:1.
- Tailing factor shall be less than or equal to 2.

3. Result & Discussion

3.1 Raw Material Characterization

3.1.1 Melting Point

Melting point was determined using digital melting point apparatus (Labatronics) by capillary method and found to be 204ºc &129°c for STG & SMV respectively. The observed melting pointcorresponding with reference value as per USP (203-206 ºC &127-130°C).

3.2 UV Method Development

It may be defined as a method of analysis that embraces the measurement of absorption by chemical species of radiant energy at definite and narrow wavelength, approximating monochromatic radiation. UV visible spectroscopy is a technique used for qualitative and quantitative determination of different kinds of analyte. Many organic molecules comprises of different types of electron which may absorb energy and reach to their corresponding excited state. The phenomenon of absorption of energy in UV region 200-400nm is the basis for quantitative as well as qualitative determination of many organic molecules. The functional group consisting of different types of electrons are responsible for absorption of energy and hence known as chromophore. Present investigation includes development of UV method for quantitative determination of STG & SMV.

From stock solution of mixture, 600µg/ml solution of STG and 60µg/ml SMV was prepared in the mixture of methanol: water (90:10). This solution was subjected to UV analysis in qualitative mode to determine the absorption maxima (λmax). The UV spectrum obtained was as given in figure and showed the absorption at different wavelengths as given in table below. The isobestic point was determined & selected for quantitative determination of STG & SMV as mentioned in further sections.

3.2.1 Determination of wavelength

For Sitagliptin Phosphate

From the following calibration U.V spectrum the wavelength maxima of STG was found to be 268nm as shown in **Figure 1**.

Table 7 Wavelength Maxima for Sitagliptin Phosphate

Sr. No.	Wavelength (nm)	Absorbance
1	268	1.352
2	238	0.164

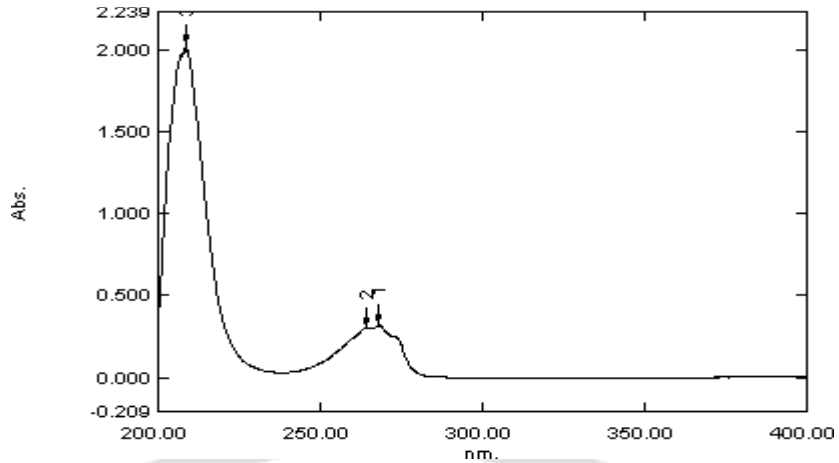

Fig 1 Calibration Curve of Sitagliptin Phosphate

For Simvastatin

From the following calibration of U.V spectrum the wavelength maxima of SMV was found to be238 nm as shown in **Figure 2**.

Table 8 Wavelength maxima for Simvastatin (SMV)

Sr. No.	Wavelength (nm)	Absorbance
1	236	1.233
2	205	0.110

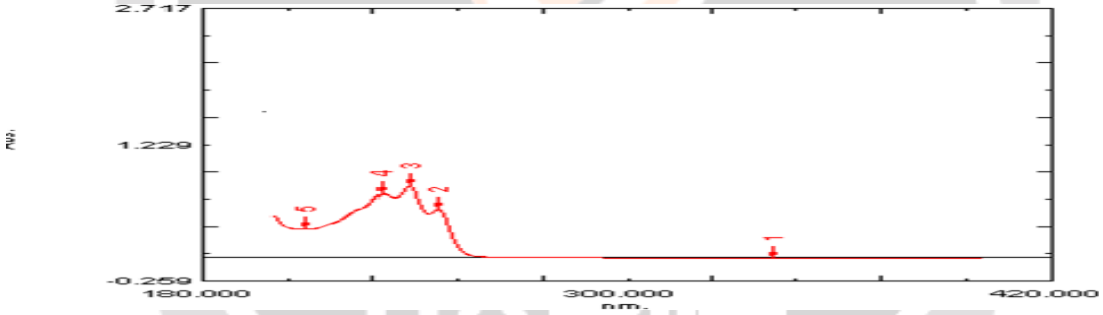

Fig 2 Calibration Curve of Simvastatin

For Mixture

The isobestic point of STG & SMV was found to be at 252 nm as shown in **Figure 3.** Hence, 252nm wavelength was selected for below validation parameters.

Table 9 Isobestic Point

Sr. No.	Wavelength (nm)	Absorbance
1	252	0.620

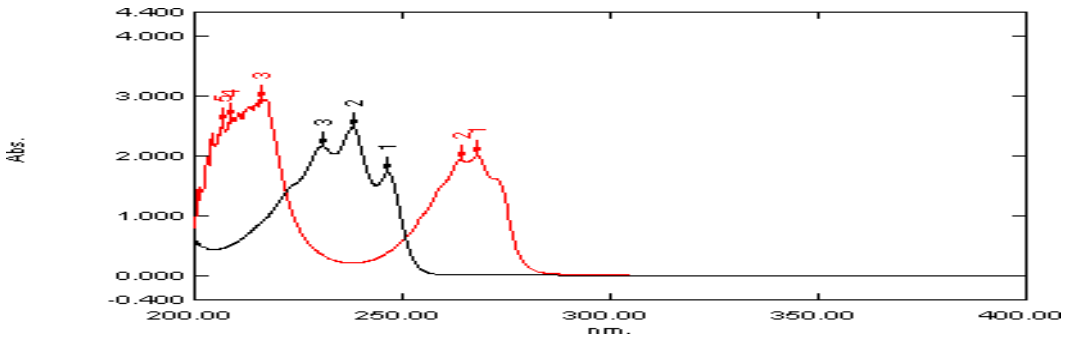

Fig 3 Calibration Curve of Mixture

3.3 Method Validation by UV Spectroscopy

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samplewithin the given range. It should be established across the range of the analytical procedure. Linearity is generally reported as the correlation coefficients, the slope of regression line i.e., r2 ≥ 0.99.

For Sitagliptin Phosphate

Table 10 Observation for Linearity (STG)

Concentration (µg/ml)	Absorbance (268 nm)
100	0.436
200	0.644
300	0.859
400	1.034
500	1.258
600	1.389

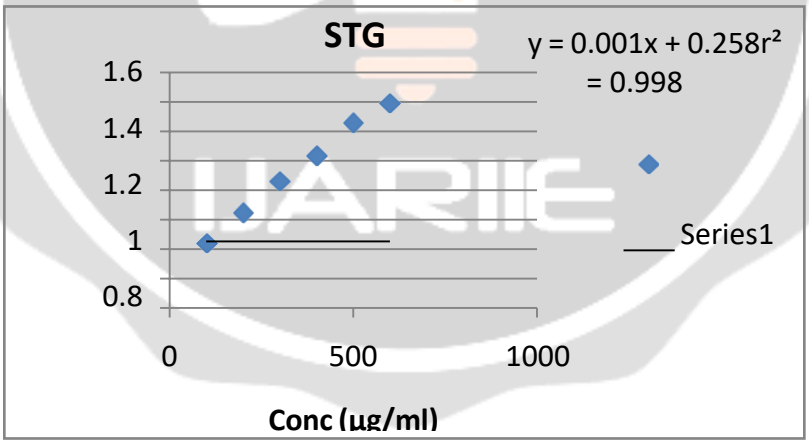

Fig 4 Linearity of Sitagliptin Phosphate

The concentration range for linearity was 100-600 µg/ml for STG. A graph was plotted with concentration on X- axis and mean absorbance on Y-axis. The r^2 value was found to be 0.998 (r^2 value should be always more than 0.99). Hence the develop method was found to be the linear in 100-600 µg/ml concentration range.

For Simvastatin

Table 11 Observation of Linearity (SMV)

Concentration (µg/ml)	Absorbance (236 nm)
10	0.147
20	0.261
30	0.353

40	0.456
50	0.567
60	0.645

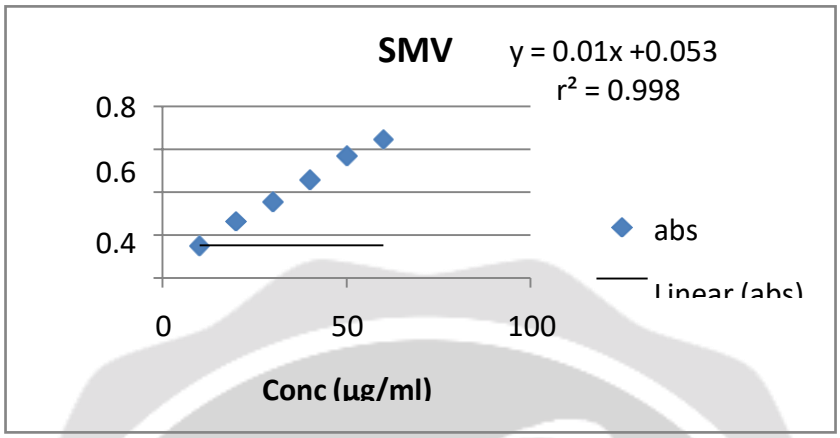

Fig 5 Linearity of Simvastatin

The concentration range for linearity was 10-60 µg/ml for SMV. A graph was plotted with concentration on X axis and mean absorbance on Y-axis. The r^2 value was found to be 0.998 (r^2 value should be always more than 0.99). Hence the develop method was found to be the linear in 10-60 µg/ml concentration array.

For Mixture

Table 12 Observation of Linearity

Concentration (µg/ml)	Absorbance (252 nm)
100	0.238
200	0.432
300	0.650
400	0.853
500	1.083
600	1.328

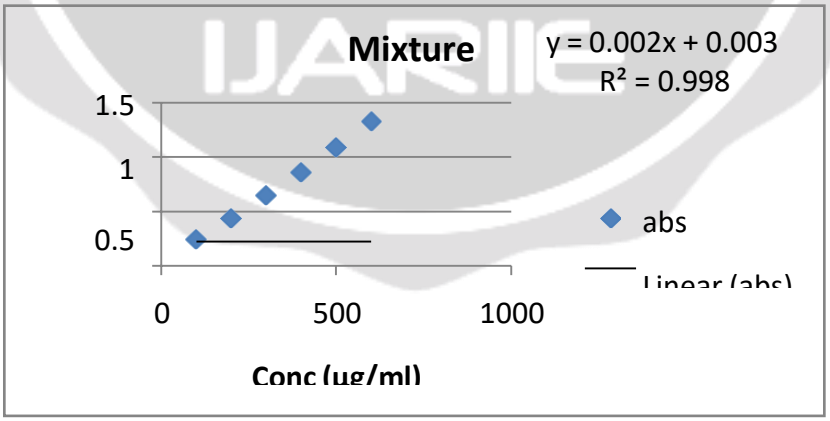

Fig 6 Linearity for Mixture

The concentration range for linearity was 100-600 µg/ml & 10-60 µg/ml for mixture .A graph was plotted with concentration on X-axis and mean absorbance on Y-axis. The r^2 value was found to be 0.998 (r^2 value should be always more than 0.99) Hence the develop method was found to be the linear in 100-600 µg/ml concentration range of mixture.

Precision

The precision of analytical method is the degree of agreement among individual test results whenthe method is applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurement. LQC is concentration which is more than lowest concentration analyzed in calibration. MQC is concentration near to middle concentration. NQC is concentration near to the highest concentration but slightly less than highest concentration. From the calibration range three QC standards were determine viz. 150, 350 and 550 µg/ml as LQC, MQC and NQC respectively. The solutions for QC standards was prepared by diluting stock solution of 1.5, 3.5 and 5.5 ml upto 10 ml. Absorbance of each QC standard was recorded for intra day and inter day precision in triplicates as per ICH guidelines Q2R1.

For Intra-Day Precision

Table 13 Intra day Precision

Sr. No.	Conc (µg/ml)	Mean abs	SD	%RSD	Inference
1	150	0.374	0.000548	0.137	Passed
2	350	0.826	0.000448	0.054	Passed
3	550	1.299	0.00068	0.052	Passed

For Inter-Day Precision

Table 14 Inter day Precision

Sr. No.	Conc µg/ml	Mean abs	SD	%RSD	Inference
1	150	0.367	0.00047	0.130	Passed
2	350	0.817	0.00056	0.069	Passed
3	550	1.296	0.00037	0.059	Passed

* mean of absorbance of seven replicate of each

The proposed method had yielded quite consistent results indicating particularity of method for quantitative determination of number of observation for STG and SMV mixture sample. Precision study illustrated that %RSD of mean absorbance of 150, 350 and 550 µg/ml were less than 2%. Therefore, the result obtained for precision study was within limit (less than 2% RSD) as per ICH guideline Q2R1.

% Accuracy

The accuracy of an analytical method is the closeness of test results, obtained by that method to the true value. The accuracy of an analytical method should be established across its range. accuracy was determined by data of precision. As per ICH guideline Q2R1 accuracy was determined at three concentration levels (QC standards) across the range. The mean absorbance was determined at said three different levels and corresponding concentration for each level was computed from Beers's law. From the observed concentrations and equivalent nominal concentrations, percent accuracy was determined using formula. Results of the same were as cited in below **Table 14**. Results obtainedwere found to be within range of standard for STG and SMV.

Table 15 % Accuracy

Sr. No.	Concentration (µg/ml)	Mean absorbance	Amt recover (µg/ml)	% Assay	Limit (97-103%)
1	150	0.371	149.6	99.73%	Passed
2	350	0.823	350.8	100.22%	Passed
3	550	1.299	552.4	100.43%	Passed

Robustness

The robustness of analytical method is the measure of its capacity, to remain unaffected by smallbut deliberate variations in method parameters and provides an indication of its reliability during normal usage. Experiments were performed for 100 µg/ml concentration of mixture by changing conditions such as wavelength (±1).

Table 16 Robustness

Sr. No.	Conc (µg/ml)	Wavelength	Mean abs	SD	%RSD	% Assay	Inference
1	100	252nm(std)	0.237	0.00044	0.188	100.0	Passed
2	100	251nm(-1)	0.242	0.00134	0.492	102.1	Passed
3	100	253nm(+1)	0.233	0.00044	0.198	98.51	Passed

*****mean absorbance of five replicates

The absorbance of 100 µg/ml of mixture was recorded at three different wavelengths viz, 251, 252 (standard) and 253 nm, Various parameter such as (mean, SD, %RSD) was found in limit as given in table. Ultimately, the percent assay values analogous to observed concentrations weredetermined. All values obtained for percent assay were in agreement with pharmacopoeial standard for STG and SMV. Therefore, the developed method was robust for deliberate variationin wavelength. %RSD for change in method parameter (i.e wavelength ±1) was found within limit (NMT 2%).

LOD & LOQ

LOD and LOQ for given method was determined from above formula, LOD is the lowest limit of analyte for given method was found to be 0.99 µg/ml and LOQ is the limit of quantificationof analyte for given method was found to be 3.00µg/ml. From the result obtained it was concluded that the concentration of mixture as less as 0.99 µg/ml can be successfully detected and concentration above 3.00 µg/ml can be productively quantified.

Table 17 LOD & LOQ

Std solution	LOD (µg/ml)	LOQ (µg/ml)
Mixture(STG &SMV)	0.99	3.00

3.4 HPLC Method Development

3.4.1 RP-HPLC Method

This technique is commonly used for quantitative estimation of drug substances from their formulation as well as from the biological fluids. This is useful for analytical study of drug molecule. Ease of performance, w specificity and analysis of sample of complex nature are important features of HPLC.

3.4.2 Selection of Analytical Column

HPLC system with Phenomenex C18 (4.6 x 250 mm, 5 µm) analytical column and UV visible detector was selected for method development. The standard and sample solutions of Sitagliptin phosphate and Simvastatin were prepared in mobile phase. Variety of HPLC grade solvents with different polarities in different proportions were tried as mobile phase for development of the chromatogram.

3.4.3 Selection of Mobile Phase

The mobile phase that was found to be most suitable for STG and SMV comprised methanol: water (90:10v/v). This mobile phase compositions provided superior resolution and most favorable retention time with appropriate tailing factor.

3.4.4 Selection of Analytical Wavelength

While selecting detection wavelengths in HPLC it is usual practice to select isobestic point of individual drugs from overlain UV spectra or select λ_{max} of standard laboratory mixture of both the drugs. The λ_{max} of standard laboratory mixture was set at 252.0 nm, at this wavelength STG showed peaks with sufficient height and also SMV exhibited comparable result parameters. Finally, from all trials the detection wavelength was selected as 252.0 nm where the peak heightof both the drugs was acceptable. The following chromatographic conditions were established by trial and error described in **Table 18** and kept constant throughout the experiment.

Table 18 Chromatographic Conditions

Column	C18 (4.6 ×250 mm)
Particle size packing	5 µm
Detection wavelength	252.0 nm
Flow rate	1 ml/min
Temperature	Ambient
Sample size	10µl
Mobile phase	Methanol: water (90:10 v/v)

3.4.5 System Suitability Testing

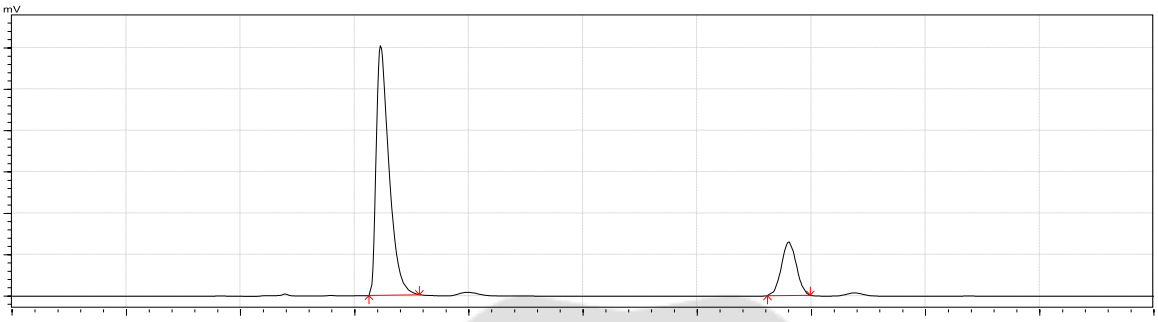

Fig 7 Chromatogram of STG & SMV

To optimize the chromatographic conditions, the effect of chromatographic variables such as composition of mobile phase, flow rate and the column were studied. The resulting chromatograms were recorded and the chromatographic parameters such as peak area, resolution and theoretical plates were integrated. The conditions obtained most excellent resolution, symmetry factor and theoretical plates were selected for further estimation. These were as shownin Table 16. The test was performed by seven replicate injections of standard working solution ofdrug. The concentration was kept constant at 400 µg/ml and 40 µg/ml for STG and SMV respectively. The best resolution and peak shape, without unnecessary tailing, were obtained by use of chromatographic conditions as stated in **Table 18**. The best resolution with reasonable retention time was obtained with mobile phase containing methanol, water (90:10) with flow rate 1.0 ml/min in low pressure gradient mode as shown in **Table 19.** A representative chromatogramwas shown in **Figure 6**. Therefore, from system suitability testing it was concluded that the system with stated chromatographic conditions (**Table 16)** would be suitable for quantitative estimation of STG & SMV at 252nm.

Table 19 System Suitability

Sr. No.	Parameter	Mean area		Limit	Inference
		STG	SMV		
1	Area	46416	12539	%RSD (<2%)	Pass
2	Retetion time	3.28	6.7	<10-5	Pass
3	Therotical plates	4615	11985	>2000	Pass
4	Tailing factor	1.7	1.06	<2	Pass

3.5 Method Validation by HPLC

Linearity

The linearity of method was estimated by preparing the aliquots of standard stock solution of STG and SMV. Each standard solution was injected to given chromatographic condition in triplicate and mean area was observed. The calibration curves were prepared by plotting the peak areas of the drugwhich were linear in the concentration range of 400-1000 µg/ml and 40-100 µg/ml for STG and SMV respectively **(Figure 7 and 8).** The correlation coefficient (r^2) was found to be 0.998 and 0.999 for STG &SMV which are in agreement as per ICH guideline (>0.990) and ensured good correlation between the peak area ratio and standard analyte concentrations.

For Sitagliptin Phosphate

Table 20 Observation of Sitagliptin Phosphate

Sr. No.	Concentration (µg/ml)	Area
1	400	46374
2	500	61540
3	600	70209
4	700	83330
5	800	95979
6	900	106873
7	1000	122010

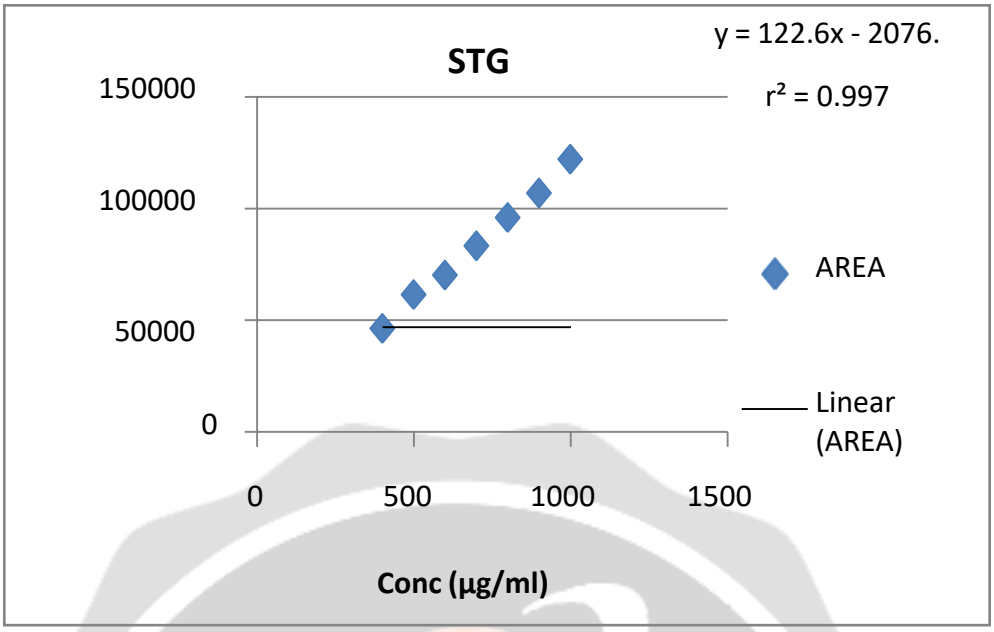

Fig 8 Linearity of Sitagliptin Phosphate

For Simvastatin

Table 21 Observation of Simvastatin

Sr.No	Concentration(µg/ml)	Area
1	40	10230
2	50	14280
3	60	17680
4	70	21722
5	80	25620
6	90	29330
7	100	32880

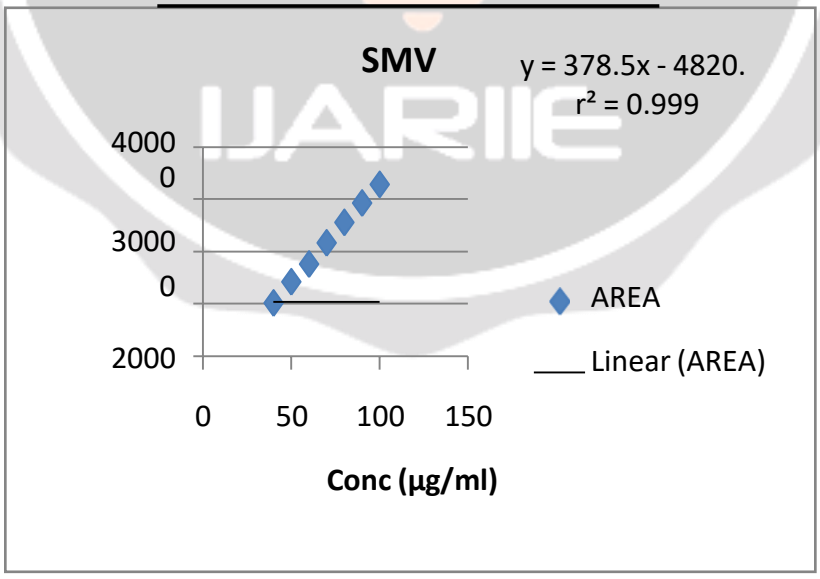

Fig 9 Linearity for Simvastatin

Precision

The precision was determined from the three QC standard as LQC, MQC and NQC. These QC standards were 450, 650, and 950 µg/ml and 45, 65and 95 µg/ml for STG and SMV correspondingly. Seven replicates for each QC standard were injected with stated chromatographic conditions and observed for various parameters namely area, retention time, tailing factor and therotical plates. The area of each QC standard for individual drug was recorded and their % RSD were calculated. All these parameters were within limit as per ICH guideline Q2R1. Hence, the method was precise for given range for both drugs.

Table 22 Precision study for STG

Sr. No.	Concentration (µg/ml)	Intra day		Inter day	
		Mean Area*± SD	%RSD	Mean Area*± SD	%RSD
1	450	57610±570.0	0.99	58054±519.6	0.89
2	550	75652±439.5	0.57	76239±226.0	0.29
3	950	111829±670.4	0.95	112078±209.8	0.86

Table 23 Precision study of SMV

Sr. No.	Concentration (µg/ml)	Intra day		Inter day	
		Mean Area*± SD	%RSD	Mean Area*± SD	%RSD
1	45	8854±128.1	1.26	13427±245.9	1.8
2	55	16380±273.0	1.08	76239±289.4	1.4
3	95	32884±344.0	1.05	112078±511.5	1.5

*****Mean area of seven replicates

Hence, from observation of **Table 21,22** it was concluded that the method followed test for precision for STG & SMV as per ICH guidelines.

% Accuracy

Accuracy was determined by data of precision study and the results obtained were as depicted in **Table 22, 23.** As per ICH guideline Q2R1 accuracy was determined at three concentration levels (QC standards) across the range. The mean areas of seven replicate injections were determined and corresponding concentration for each level was computed from regression equation. Fromthe measured concentrations and correspondent nominal concentrations, percent accuracy was determined using above formula. Results of the same were mentioned in **Table 24, 25**. Results attained were found within range of pharmacopeial standards for STG &SMV.

Table 24 % Accuracy of STG

Sr. No.	Concentration (µg/ml)	Meanarea	Amount recover(µg/ml)	% Assay	Limit (98-103%)
1	450	58009	460	102.2	Passed
2	650	76285	647	99.53	Passed
3	950	109871	955	100.5	Passed

Table 25 % Accuracy of SMV

Sr. No.	Concentration (µg/ml)	Meanarea	Amount recover(µg/ml)	% Assay	Limit (97-103%)
1	45	7698	46.19	102.6	Passed
2	65	15316	64.04	99.07	Passed
3	95	32623	96.12	101.1	Passed

Robustness

Table 26 Robustness for Flow rate

Sr. No.	Flowrate (ml/min)	Mean of Area		Mean of RT		% Assay		Limit(97-103%)
		STG	SMV	STG	SMV	STG	SMV	
1	1(std)	59968	13280	3.2	6.7	100	100	Passed
2	1.05(+1)	57907	11420	3.09	6.61	98.50	99.84	Passed

3	0.95(-1)	61963	15342	3.3	6.83	102.01	101.9	Passed

*****mean area of seven replicates was found within limit

Table 27 Robustness for Mobile Phase

Sr. No.	Mobile Phase ratio	Mean of Area		Mean of RT		% Assay		Limit(97-103%)
		STG	SMV	STG	SMV	STG	SMV	
1	90:10(std)	60220	13130	3.2	6.7	100	100	Passed
2	91:09(+)	62012	15064	3.16	6.78	98.18	101.1	Passed
3	89:11(-)	59326	12045	3.13	6.65	98.05	99.75	Passed

The robustness was studied by analyzing the sample of STG and SMV at 500 and 50 µg/ml concentration by intentional dissimilarity in method parameters and the change in the response ofSTG and SMV were noted. The method parameters in which deliberate fluctuations were made include mobile phase composition (±1 %), flow rate (±0.5 ml/min). The mean area and mean RT of 500 and 50 µg/ml of STG & SMV were recorded in seven replicates. The percent assay valuescorresponding to observed concentrations were determined as stated in **Table 26, 27**. All values obtained for percent assay were in agreement with pharmacopeial standard for STG & SMV. Therefore, the developed method was found to be robust as the results were not significantly affected by slightly variation in chromatographic parameter. Hence developed method would be robust during normal usage.

LOD & LOQ

Limit of detection and limit of quantification were established by using formulaLOD = 3.3× δ/S and LOQ=10×δ/S (Where S was slope of calibration curve and δ was the standard deviation of area in calibration plot). LOD was found to be 0.85 & 0.3 µg/ml for STG and SMV. LOQ was found to be 2.58 & 0.99 µg/ml for STG and SMV correspondingly shownin **Table 28**. Therefore, the concentration of both drug as low as 0.85 & 0.3µg/ml could be detected and 2.58 and 0.99 µg/ml could be effectively quantified without any disturbance of impurity.

Table 28 LOD & LOQ

Parameter	STG (µg/ml)	SMV(µg/ml)
LOD	0.85	0.3
LOQ	2.58	0.99

% Recovery

The recovery experiment was carried out by spiking the standard sample of 500 and 50 µg/ml STG and SMV respectively to the test solutions prepared from finished product at 80, 100 and 120 % levels. Recovery study was carried out by standard addition method in which known amount of standard solution of STG and SMV (500 and 50 µg/ml) were added to each level of test solution as stated above. The resultant solution were injected in triplicates and observed for area obtained for individual drug. Mean area of standard drug sample was substracted from the area obtained for each level to obtain the actual area corresponding to test sample. From the measured area the amount recovered in percent for each STG and SMV was determined. The results obtained were in agreement to compendial standards of individual drug, **Table 29, 30.** Eventually, it was concluded that the developed simultaneous method can be explored for routineanalysis of STG and SMV. The recovery result was shown in chromatogram **Figure 10.**

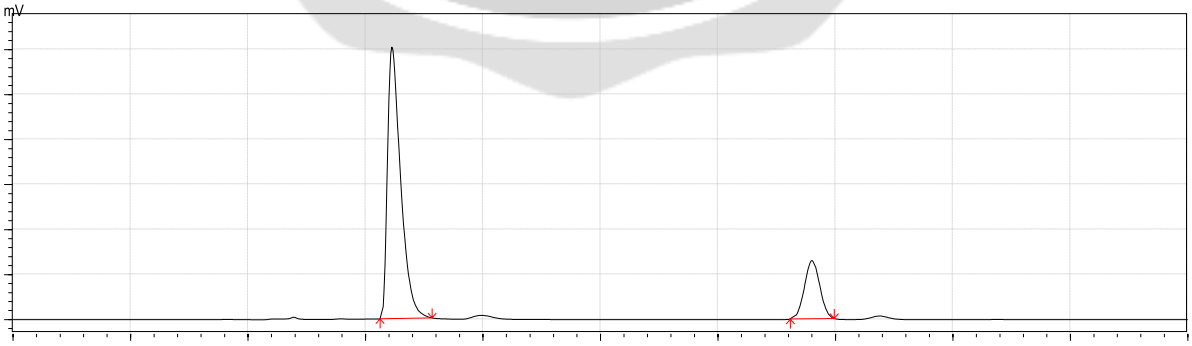

Fig 10 Chromatogram for recovery study of STG & SMV

Table 29 For STG

Recovery level	Concentration	Amt added	Amt found	%	Limit (97-

	(µg/ml)	(µg/ml)	(µg/ml)	Recovery	103%)
80%	500	480	994.1	101.42	Passed
100%	500	500	998.0	99.80	Passed
120%	500	520	994.5	98.01	Passed

Table 30 For SMV

Recovery level	Concentration (µg/ml)	Amt added (µg/ml)	Amt found (µg/ml)	% Recovery	Limit (97-103%)
80%	50	48	98.79	100.81	Passed
100%	50	50	101.78	101.78	Passed
120%	50	52	100.25	99.00	Passed

4. CONCLUSIONS

The present study was aimed at develop a sensitive, precise and accurate HPLC method for the simultaneous analysis of Sitagliptin phosphate and Simvastatin in bulk drug and in combined pharmaceutical dosage forms. In order to affect analysis of the component peaks, mixture of methanol and water was selected mobile phase on a C18 stationary phase. A mixture of methanoland water in the ratio of 90:10 v/v was proved to be the most suitable for combination since the chromatographic peaks were better defined and resolved and almost free from tailing. The retention times of Sitagliptin phosphate and Simvastatin were found to be 3.2 and 6.7 min respectively. Each of the samples was injected seven times and the same retention times were observed in all cases. A good linear relationship was observed between the concentration of Sitagliptin phosphate and Simvastatin their respective peak areas. The regression curves were constructed by linear regression fitting and mathematical expressions of STG and SMV. High recovery values obtained from the combined dosage form by the proposed method indicated the method was accurate. The absence of additional peaks indicates non-interference of common excipients used in the tablets.

System suitability parameters were studied with seven replicate standard solution of the drug andthe calculated parameters are within the acceptance criteria. The tailing factor and the number theoretical plates are in the acceptable limits.

The deliberate changes in the method had no much effect on the peak tailing, theoretical plates and the percent assay. This indicated that the present method was robust. The lowest values of LOD and LOQ as obtained by the proposed method indicate the method was sensitive. Hence weconcludes that the proposed HPLC method was sensitive and reproducible for the simultaneous analysis of Sitagliptin phosphate and Simvastatin in combined pharmaceutical dosage forms withshort analysis time

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