ANALYTICAL METHOD DEVELOPMENT & VALIDATION OF ANAGLIPTIN BY RP-HPLC

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ABSTRACT

Analysis is important in every product but it is vital in medicines as it involves life. The assurance of quality is achieved through analysis of the drug product. Now a days, the antidiabetic drugs are very much useful in single therapies, rather than the use of multiple drug formulation because of multiple action side effects. Aanagliptin is a life saving molecules for the treatment of Type II Diabetes. Quantification of drug molecule is important task for routine analysis of API in its finished product analysis. Literature survey exposed very few analytical procedure for routine analysis of Anagliptin. Therefore, in present research an attempt was made to develop and validate, simple, precise and economic method for routine analysis of Anagliptin in bulk and tablet dosage form. It was competed that method would be consistent for quantification of Anagliptin throughout its lifecycle.

Keyword: - *Anagliptin, HPLC, Antidiabetic drug etc….*

1. Introduction

Chromatography was first invented by Michael Tswett, a Russian botanist in 1906 for the separation of colored substance into individual component. 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.

1. UV-visible Spectrophotometry

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.

$$A = log\ Io/It = abc$$

Equation 1 Beers Lamberts law

Where,
A = Absorbance of the solution at particular wavelength of the light beam
Io = Intensity of incident light beam
It = Intensity of transmitted light beam
a = Absorptivity of molecule at the wavelength of beam
b = Path length of cell in cm

c = Concentration of solution in (gm/lit).

2. High performance liquid chromatography (HPLC).

HPLC is an analysis technique that yields high performance and high speed compared with traditional column chromatography because of the forcefully pumped mobile phase. Recently, ultrafast analysis using a high-pressure-resistant apparatus has been attracting attention. UHPLC(Ultra High Performance Liquid Chromatography) technique is based on the same method of separation as classical column chromatography.

2.1 Types of HPLC

A) Normal-phase chromatography

B) Reversed-phase chromatography

Table 1 Normal vs. Reversed Phase Chromatography

Content	**Normal**	**Reverse**
Packing polarity	High	Low
Solvent polarity	Low	High
Elution order	Nonpolar first	Polar first
Effect of increasing solvent polarity	Decrease RT of polar drug	Increases RT of non-polar drug

Characteristics to be validated in HPLC and their Acceptance criteria

Characteristics	**Acceptance criteria**
Accuracy	Recovery 98-102% (individual) with 80
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate Precision	RSD < 2%
Specificity / Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	Correlation coefficient r > 0.999
Range	80 –120 %

2. AIM AND OBJECTIVES

2.1 AIM

To develop and validate, simple, precise and economic method for routine analysis of Anagliptin in bulk and tablet dosage form.

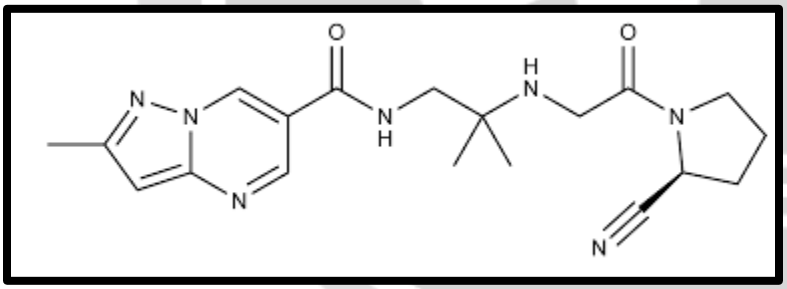
It was competed that method would be consistent for quantification of Anagliptin throughout its lifecycle.

2.2 Objectives

- To develop a new UV Spectrophotometric method.
- To validate UV Spectrophotometric method as per ICH guideline.
- To develop a new, LC compatible, simple, precise, accurate, rapid & cost effective RP-HPLC method for estimation of Anagliptin in bulk and tablet dosage form.
- To validate RP-HPLC method as per ICH guideline.

3. DRUG PROFILE

Name: Anagliptin



ChemicalName:N-[2-[[2-[(2S)-2-Cyanpyrollidin-1-yl]-2-oxoethyl]amino]-2-methylpropyl]-2-methylpyrazolo[1,5-a]pyrimidine -6-carboxamide

Molecular formula: $C_{19}H_{25}N_7O_2$

Molecular weight: 383.45 gm/mol

Solubility: Acetonitrile, Methanol, Water

Category: Dipeptidyl peptidase-4 (DPP-4) inhibitor

Wavelength: 248nm

Mode of action: Anagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor. It used in Non- insulin dependent type-2 diabetes mellitus and approved in Japan for treatment.

4. RAW MATERIAL CHARACTERIZATION

5.1 Characterization of Anagliptin ethanolate

Determination of melting point

Melting point was determined using digital melting point apparatus. The reference melting point of Anagliptin is 101 0C

2 Reagents and chemicals

Table 4 chemicals and reagents

Sr.No.	Chemical/Reagent or Solvent	Grade	Make	Characterization
1	Acetonitrile	HPLC	Research lab	As HPLC grade no further characterization performed
2	Water	HPLC	-	As HPLC grade no further characterization performed

3 Determination λ max by UV

100µg/ml solution of Anagliptin(ANA) was prepared by accurately weighing 10mg of ANA. It was then transferred to 100ml volumetric flask containing few ml of acetonitrile. Finally the volume was made upto the mark using water. The resultant solution was scanned using UV visible spectrophotometer in the range of 200-400nm. The reference λmax ofANA is 247nm.

5. Experimental work

5.1 UV method development and validation

Determination of Wavelength Maxima

Preparation of standard stock solution

10mg of ANAwas weighed accurately and transferred to a 100ml volumetric flask containing some amount of solvent(acetonitrile). Volume was made up to the mark using water to obtain the resulting solution of 100µg/ml. The absorbance of the latter was recorded using UV visible spectrophotometer in range 200-400nm.Similarly 100 ug/ml solutions of ANA were prepared in 18:82acetonitrile: water. The absorbance of each of these solutions was recorded by using UV spectrophotometer (Shimadzu 1800).

Study of Beer – Lambert's law

The aliquot portions of stock standard solutions of ANA were diluted appropriately with mobile phase to get a series of concentration between 5-30ppm of drug and concentration were scanned at 248 nm..

5.2 Method optimization

Preparation of working solution

Accurately weighed 10mg of ANA and transferred to 100ml volumetric flask containing a mixture of acetonitrile and water in the ratio of 18:82.The volume was made upto the mark using same mixture of solvent then 1.0 ml was pipetted out and diluted upto 10ml which will give resultant solution of 10µg/ml which was used for optimization. five replicates of the solution were performed and absorbance was recorded at 248nm. Mean SD and %RSD were calculated.

5.3 Method development and Validation-

The analytical method was validated with respect to parameters such as linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, robustness, and recovery(as per ICH Q2(R1), 2005).

Linearity-

Linearity was established by least squares linear regression analysis of the calibration curve.

Accuracy-

Accuracy was studied by adding two different amounts (corresponding to 80%, 100% and 120% of the test preparation concentrations) of Anagliptin. For each level, three solutions were prepared and each was injected in duplicate.

Precision-

The precision of the method, as intra-day repeatability was evaluated by performing six independent assays of the test sample preparation and calculating the RSD %. The intermediate (interday) precision of the method was checked by performing same procedure on different days by another person under the same experimental conditions.

LOD and LOQ-

The LOD and LOQ of Anagliptin were calculated by mathematical equation:

LOD = 3.3 * standard deviation / slope(m)

LOQ = 10 * standard deviation / slope(m).

Robustness-

Robustness of proposed method was performed by changing UV analyst and remaining conditions (solvent, dilution, UV Spectrophotometer) were same.

6. HPLC METHOD DEVELOPMENT AND VALIDATION

From results obtained from UV method acetonitrile: water (18:82) was selected as mobile phase for HPLC method development.

6.1 Preparation of stock solution

Accurately weighed 10mg of ANA and transferred to 10ml volumetric flask containing a mixture ofacetonitrile: water (0.1%FA) (18:82). The volume was made upto the mark using same mixture of mobile phase. The resulting stock solution was filtered through 0.45µ membrane filter and sonicated for three cycles each of 10 min.

6.2Chromatographic parameters

Column :	**Phenomenex C18 (250mm×4.6mm), 5µm**
Flow Rate :	1 ml/min
Wavelength :	248 nm
Injection volume :	1µl
Column oven temperature :	Ambient (25^0c)
Run Time :	10 minutes

6.3 System suitability testing

Preparation of working solution

0.1 stock solution was pipetted out and diluted upto 10ml to obtain consequential solution of 10µg/ml. The resulting solution was filtered through 0.45µ membrane filter and sonicated for three cycles each of 10 min. three replicates of this solution were injected and results were recorded for RT, 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.

The column efficiency for ANA peak should not less than 2000 theoretical plates.

The tailing factor for peak, should not more than 2.0.

% CV for area shall NMT 1.5 and for RT NMT 0.5%

6.4 Method Validation

A) Linearity

From stock solution 0.1 , 0.2 , 0.3 , 0.4 , 0.5were pipetted out and diluted upto 10 ml to obtain 10, 20, 30, 40, 50µg/ml resultant solutions respectively. Calibration curve was constructed between concentrations versus peak area. Results wererecorded for equation of line, correlation coefficient and intercept were determined.

Y= mX+c

Equation 2 Slope equation

Where, Y- area

X- Unknown concentration

m- Slope of graph

c- Intercept

B) Precision

From the calibration range three QC standard decided viz. 20, 30 and 40µg/ml as LQC, MQC and NQC respectively. The solutions for QC standards were prepared by diluting stock solution of 0.2,0.3, and 0.4ml solutions upto 10ml. Area of each QC standard were recorded for intraday and interday precision in three replicates as per ICH guidelines Q2R1. Results were recorded to calculate mean, SD, %RSD.

C) % Accuracy

% Accuracy was determined from the observations of precision study using following formula. Limit for % accuracy is NMT 5% RSD.

$$\%\ Accuracy = Mean\ measured\ conc - \frac{Nominal}{Nominal} \times 100$$

Equation 3 % accuracy equation

D) Robustness

5µg/ml solution was selected for robustness study for the parameters like mobile phase ratio, flow rate, wavelength 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.

Condition	Normal	Variation 1	Variation 2
Mobile phase	82 : 18	81:19	83:17
Flow rate	1ml/min	1.05ml/min	0.95ml/min

Table 5 Robustness variation table

E) %Recovery

Preparation of stock from API

Accurately weighed 10mg of ANA (API) was added in volumetric flask containing some amount of mobile phase and volume was made upto 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 1.0ml of stock was pipetted out in triplicate and kept in three different volumetric flasks, cleaned previously and diluted upto 10ml by using mobile phase to obtain

resultant solution of 10µg/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 300mg of ANA) were weighted, average weight was determined and powdered. Powder equivalent to 10mg (21.4mg) 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 0.8, 1.0, 1.2ml solutions were pipetted out and diluted upto 10ml using mobile phase to obtain resultant solution of 8, 10 and 12µg/ml.

Preparation of test solution for % recovery by spike method

10µg/ml solution of ANA (API) was spiked into each of above dilutions of 8, 10 and 12µg/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.

$$\%recovery = \frac{sample\ area}{standard\ area} \times \frac{standard\ dilution\ factor}{sample\ dilution\ factor} \times \frac{average\ weight}{label\ claim} \times 100$$

Equation 4 % accuracy equation

7. RESULTS AND DISCUSSION

Raw material characterization

Melting point

Melting point was determined using digital melting point apparatus (Labatronics) by capillary method and found to be 101º C.The observed melting point corresponding with reference value as per USP (102º C).

Characterization of reagent and chemicals

All regents and chemicals used were of HPLC grade and hence no further characterization was done.

7.1 UV method development

UV Visible spectroscopy is a tool 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 ANA.Initially, 30µg/ml solution of ANAwas prepared in the mixture of acetonitrile: water (18: 82). 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 wavelength of 248nm was selected for quantitative determination of ANA as given in further sections.

7.1.1 Determination of Wavelength

Table 6 Determination of Wavelength

Sr.No.	Wavelength(in nm)	Absorbance
1	248.40	0.594
2	229.60	0.042

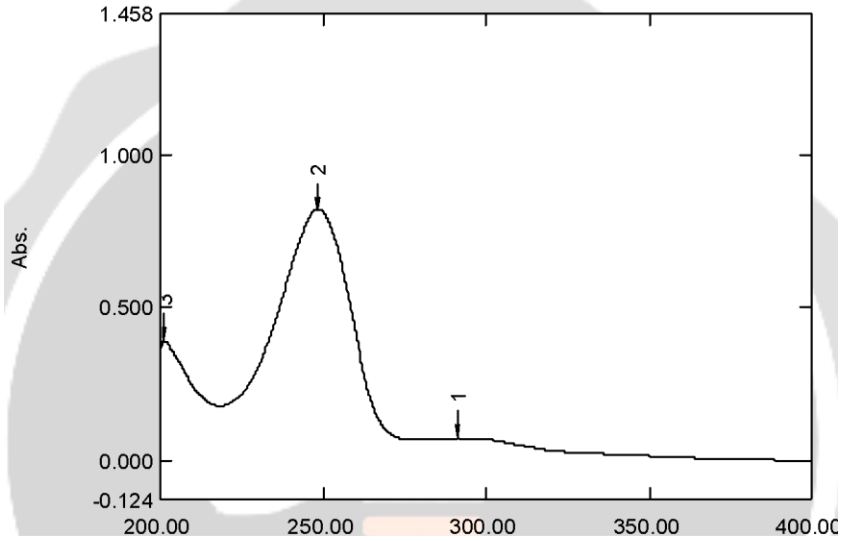


Figure 3 Wavelength maxima for Anagliptin

From the above calibration U.V spectrum the wavelength maxima for ANA was found to be 248nm.

7.1.2 Validation parameters

A) 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 conc. of analyte in sample within 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, $r^2 \geq 0.999$.

Table 7 Observation of Linearity

Concentration(µg/ml)	Absorbance(at248 nm)
2	0.253
4	0.471
6	0.708
8	0.983
10	1.197

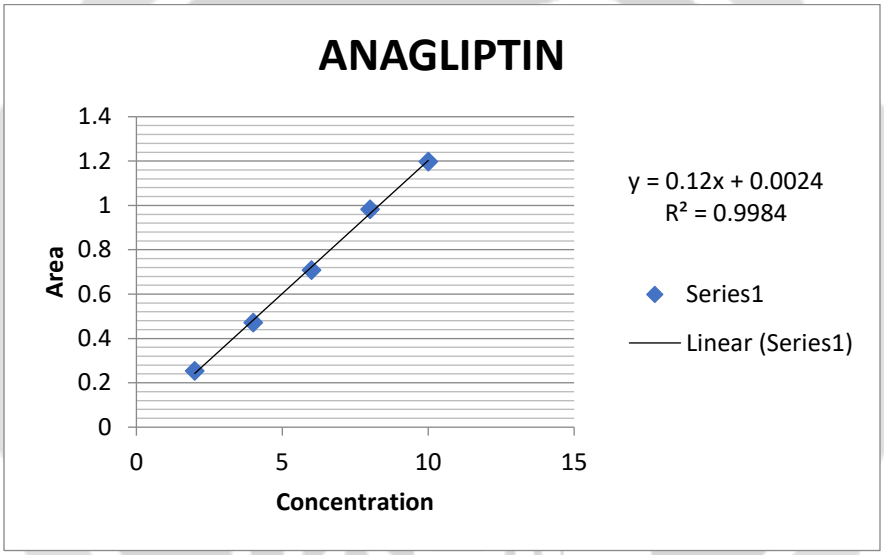


Figure 4Plot of Linearity for ANA at 247 nm

Linearity of method was ranging from concentration 2 to 10µg/ml for ANA.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 2 to 10µg/ml concentration array.

B) Precision

The precision of analytical method is the degree of agreement among individual test results when the method was applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical method was usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. From the calibration range three QC standards were determine viz. 8, 18 and 28 µg/ml as LQC, MQC and NQC respectively.LQC was the concentration more than the lowest concentration of linearity; MQC and NQC were concentration minor more than the lowest, near to middle and near to highest that of linearity. The solutions for QC standards were prepared by diluting stock solution of 0.8, 1.8 and 2.8 ml up to 10ml. Absorbance of each QC standard was recorded for intra-day and interday precision in triplicates as per ICH guidelines Q2 R1.

Table 8 For intra-day precision

Sr.No.	Concentration (µg/ml)	Mean absorbance	SD	%RSD	Inference
1	4	0.359	0.04	1.3	Pass
2	6	0.547	0.04	0.2	Pass
3	8	0.732	0.02	0.2	Pass

Table 9 For inter day precision

Sr.No.	**Concentration (µg/ml)**	**Mean absorbance***	**SD**	**%RSD**	**Inference**
1	4	0.357	0.004	0.6	Pass
2	6	0.551	0.004	0.7	Pass
3	8	0.751	0.008	0.7	Pass

***mean of absorbance of five replicate of each.**

The proposed method had yielded quite consistent results indicating particularity of method for quantitative determination of number of observation of ANA sample. Precision study illustrated that %RSD of mean absorbance of 4 ,6 , 8, ppm were less than 2%. Therefore, the result obtained for precision study was within limit (less than 2% RSD) as per ICH guideline Q2R1.

C) % 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 study and the results obtained were as depicted in table 10. 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 Beer's law. From the observed concentrations and equivalent nominal concentrations, percent accuracy was determined using formula. Results of the same were as cited in **Table 10**. Results obtained were found to be within range of pharmacopoeia standard for ANA.

Table 10 %accuracy

Sr.No.	Concentration (PPM)	Mean absorbance	Amount recover (mean measured concentration)	% Assay	Limit (95-105%)
1	4	0.315	3.8	100.5	pass
2	6	0.560	6.2	102.9	pass
3	8	0.742	7.9	100.7	pass

D) Robustness

The robustness of analytical method is the measure of its capacity, to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Experiments were performed for 10 ppm concentration of ANA by varying conditions such as change in analyst other conditions were same.

Table 11 for robustness

Sr.No.	Concentration (µg/ml)	Wavelength(nm)	Mean absorbance	SD	%RSD	Inference
1	10	248(std)	1.194	0.000577	0.13	Pass
2	10	247(-1)	1.182	0.000192	0.044	Pass
3	10	249(+1)	1.198	0.044	0.13	Pass

The absorbance of 10 ppm ANA solution was recorded in five replicates at three different wavelengths viz.247, 248 (standard wavelength) and 249 nm as given in **Table 11**. Mean absorbance at each varied wavelength was recorded and statically analyzed to determine SD and % RSD; both of these statistical parameters were found in limit as given in **Table 11**. Observed concentrations were determined from mean absorbance values using Beer's law. Eventually, the percent assay values analogous to observed concentrations were determined. All values obtained for percent assay were in agreement with pharmacopoeial standard for ANA. Therefore, the developed method was robust for deliberate fluctuation in wavelength. %RSD for change in method parameter (i.e wavelength ±1) was found within limit. (NMT 2%).

E) LOD and LOQ

LOD is defined as lowest concentration of analyte likely to be reliably distinguished from blank and at which detection is feasible and within limits. LOD was determined by following formula and found to be 0.25 µg/ml. similarly, LOQ was calculated by using below equation and was found to be 0.76 µg/ml. from the result obtained it was concluded that the concentration of ANA as less as 0.25 µg/ml can be successfully detected and concentration above 0.76 µg/ml can be productively quantified.

Table 12 LOD and LOQ

Std Solution	LOD(µg/ml)	LOQ(µg/ml)
ANA	0.25	0.76

7.2 HPLC RESULT

7.2.1 RP-High performance liquid chromatography (HPLC) method

This technique is commonly used for the quantitative estimation of the drug substances and drug product as well as for studying their metabolites. In addition HPLC as become more essential technique for estimation of drugs in biological fluid. This help analytical chemistry study pharmacodynamic fate of drug molecule in vivo.This method also offers advantages of estimating the constituents for the multicomponent system. This technique was employed in the present investigation for estimation of ANAin bulk and tablet dosage form. Various parameters influencing analysis considered an important aspect for the development of analytical method. In order to establish RP-HPLC method the different parameters were studied in further sections.

7.2.2 HPLC column and detector

HPLC system with Phenomenex C18 (4.6 x 250 mm, 5 µm) analytical column and UV Visible detector were selected for quantification and detection of ANA respectively. used for the study. The standard and sample solutions of ANA were prepared in mobile phase. Different HPLC grade solvents of varying polarities in with divers proportions were attempted as mobile phase for development of the chromatogram.

7.2.3 Selection of mobile phase

Mobile phase consisting of Acetonitrile and water(0.1% FA) in the ratio of 18:82 was selected after prior experiment runs of ANA. The detector was set at248 nm in order to obtain maximum response with in short period of time. The selection of the wavelength was based on the λmax obtained by scanning of standard solution by UV spectroscopy. This instrument settings obtained finest resolution and optimum retention time with appropriate tailing factor (<2).Chromatographic conditions were established by trial and error as given in table and were kept constant throughout the stud

Column :	Phenomenex C18 (250mm×4.6mm), 5µmparticle size
flow Rate :	0.95 ml/min
Wavelength :	248 nm
Injection volume :	1µl
Run Time :	6 minutes

7.2.4 System suitability test

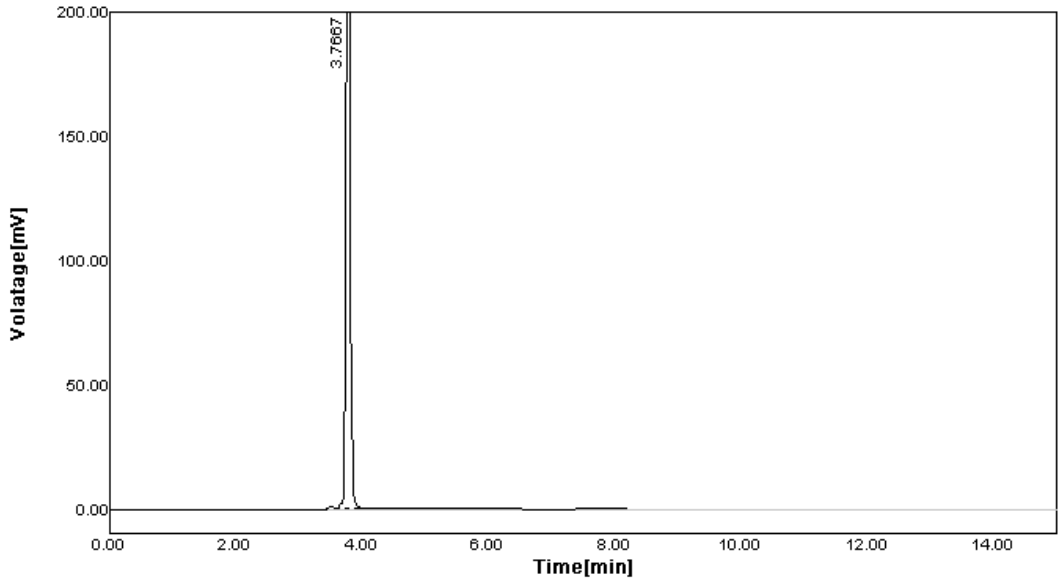


Figure 5 Separation of the drugs in selected mobile phase showing R.T. of 3.2 ANA,

Table 13 System suitability

Sr.No.	Parameter	Mean	Limit	Inference
1	Area	1315	%RSD (<2%)	Pass
2	Retention time	3.766	< 10-5	Pass
3	Therotical plates	17701	>2000	Pass
4	Tailing factor	1.333	<2	Pass

System suitability is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by seven replicate injections of standard solutions of ANA, however, the concentration was kept constant at 5 ppm. The results obtained at each replicate injection were assessedfor parameter like area, retention time, therotical plates and tailing factor and evaluated against standard pharmacopoeial limit and found within limit. The mean, SD and %RSD for area and retention time calculated as depicted in **Table 13**. The results were in agreement with the limit as per ICH guideline. Therefore from system suitability experiment it was concluded that the system was found to be suitable for quantative estimation ofANA with acetonitrile: water as mobile phase and at 248 nm wavelength.

7.2.5 Validation parameter

A) Linearity

The linearity of a test procedure is its ability (within a given range) toobtain test results proportional to the concentration (amount) of analyte inthe sample.

Table 14 linearity

Sr.No.	Concentration (µg/ml)	Area
1	10	1711.27
2	20	3327.56
3	30	5003.17
4	40	6782.37
5	50	8140.71

* the results were mean area of three replicates injections

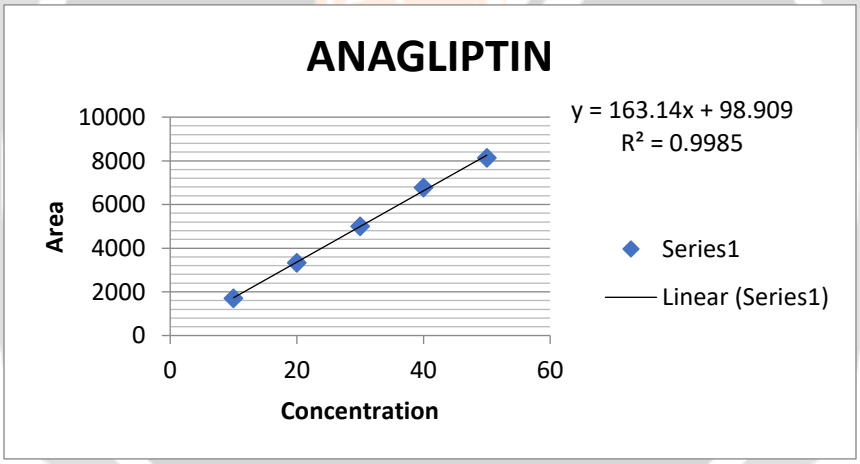


Figure 6.Plot of linearity

Linearity of method was assessed by diluting aliquots of standard stock solution of ANA to obtained 10 to 50 µg/ml solutions. Each standard solution was injected to give chromatographic system in triplicates and mean area was recorded. A calibration curve was plotted with concentration on X axis and mean area on Y-axis. The correlation coefficient, slope, intercept and equation of line were determined and found to be 0.998(r^2 value should be always more than 0.99), and Y=163.1x+98.90 respectively. Consequently, the method was found to be linear for 10 to 50 µg/ml concentration range as per ICH guideline.

B) Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision was determined from

the threeQC standards define previously as LQC, MQC, NQC. These QC standards was 20 , 30 , and 40 ppm concentrations of ANA standard solutions. Three replicates for each QC standard were performed injected for given instrument setting. The results were recorded for area, retention time, therotical plates, and USP symmetry factor and found to be in agreement with each other. The area for each QC standard was statistically evaluated for standard deviation and percent RSD as illustrated in **Table 15**.

The percent RSD obtained was in conformity with the ICH guideline. As a result, it was concluded that the method was precise for the given range.

Table 15Precision

Sr. No.	Intra day			Inter day	
	Concentration (µg/ml)	**Mean Area*±SD**	**%RSD**	**Mean Area*SD**	**%RSD**
1	20	3336.41±28.92	1.4	17772±120.8	0.87
2	30	4920.52±15.56	0.2	36888±92.2	0.32
3	40	6733.68±39.70	0.8	58936±194.2	0.59

***Mean area of Three replicates injections.**

C) LOD and LOQ

The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated under the stated experimental conditions.The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. The sensitivity of the method was estimated in terms of limit of detection and limit of quantification by using formula LOD=3.3× δ/S and LOQ=10×δ/S (Where S was slope of calibration curve and δ is the standard deviation of area in calibration plot). LOD and LOQ were found to be 1.000µg/ml and 3.053 µg/ml respectively. Consequently, the concentration of ANAas low as 1.000µg/ml can be detected and 3.053µg/ml can beeffectively quantified without any disturbunce of impurity.

Table 16 For LOD and LOQ

Std Solution	LOD (µg/ml)	LOQ(µg/ml)
ANA	1.000	3.053

D) %Accuracy

The accuracy of an analytical method is the closeness of test results, obtained by that method to the true value. Accuracy was determined by data of precision study and the results obtained were as depicted in **Table 17**. As per ICH guideline Q2R1 accuracy was determined at three levels by standard addition method (QC standards) across the range. The mean area of three replicate injections was determined at said three different levels and corresponding concentration for each level was computed from calibration curve. From the experimental concentrations and correspondent nominal concentrations, percent accuracy was determined using following formula. Results of the same were as cited in **Table 17**. Results attained were found to be within range of pharmacopoeialstandards for ANA.

$$\% \, Accuracy = Mean \, measured \, conc - \frac{Nominal}{Nominal} \times 100$$

Table 17. % Accuracy

Sr.No.	Concentration	Mean area	Amount recovered(µg/ml))	%Assay	Limit(98-102%)
1	80	3018.165	7.86	98.25	Passed
2	100	3358.625	9.98	99.86	Passed
3	120	3669.535	11.86	98.84	Passed

E) Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage

Table 18 For mobile phase

Sr.No.	Mobile phase ratio	Concentration µg/ml	Mean area	% Assay	Limit(98-102%)
1	18:82(std)	10	1711.27	100	Passed
2	19:81(±1)	10	1627.9	99.37	Passed
3	17:83(±1)	10	1793.40	100.6	Passed

Table 19 For flow rate

Sr.No.	Flow rate ratio	Mean of area	%Assay	Limit(98-102%)
1	0.95 ml/min (std)	1711.27	100.2	Passed
2	1.05 (+0.10)	1799.28	101.1	Passed
3	0.85 (-0.10)	1820.31	104.4	Passed

Experiments were performed for 10 ppm concentration of ANA by varying conditions such as mobile phase ratio (±1) and flow rate ratio (±0.10).The mean area and mean RT of 10ppm ANA solution was recorded in three replicates for deliberate change in method parameter viz, flow purposeful rate (0.85and 1.05 ml, **Table 19**) and mobile phase

ratio(18:82 , and 17:83,**Table 18**).the percent assay values analogous to observed concentrations were determined. All values obtained for percent assay were in agreement with pharmacopoeial standard for ANA. Therefore, the developed method was robust for deliberate rise and fallin mobile phase ratio and flow rate.

F) % Recovery

Table 20 %Recovery

Recovery level (%)	Concentration taken (µg/ml)	Amount spiked (µg/ml)	Amount recovered (µg/ml)	% recovery	Limit (98-102%)
80	10	8	17.90	98.20	Passed
100	10	10	19.98	99.86	Passed
120	10	12	21.86	101.58	Passed

Percent recovery is determination of percent purity of given analyte finished product.The accuracy of the methods was determined by calculating recoveries of ANA by thestandard addition method.Known amount of standard solutions of ANA (8, 10 and 12µg/ml)were added to a preanalysedsample solution of ANA (10µg/ml).Each solution was injected intriplicate and the percentage recovery wascalculated by measuring the peak areas and fittingthese values into the regression equations of thecalibration curves. Data obtained is represented in **Table 20**. The proposed method afforded the percent recovery of ANA as per pharmacopoeial limit.

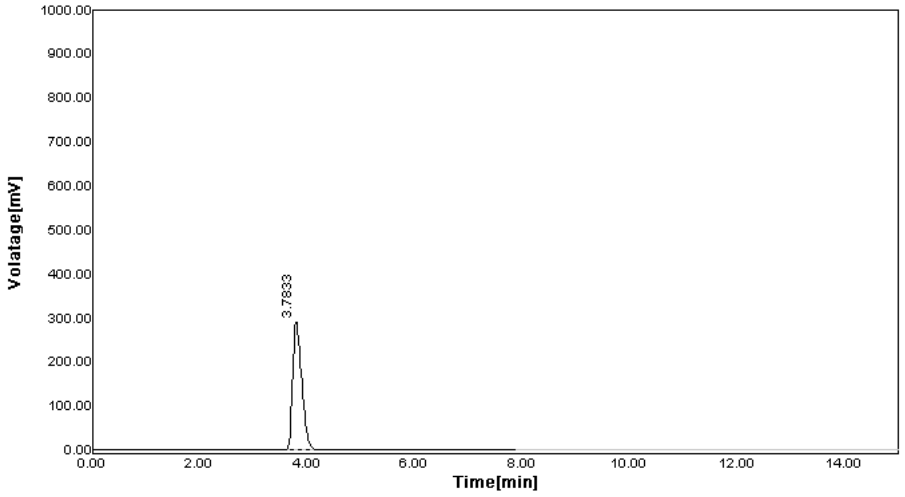


Figure 7 Graph of % recovery for marketed formulation

8. SUMMARY AND CONCLUSION

The present study was aimed at developing a sensitive, precise and accurate HPLCmethod for the analysis of Anagliptinin bulk drug and in pharmaceutical dosage forms. Inorder to affect analysis of the component peaks, mixtures of acetonitrile with water in different combinations were tested as mobile phase on a C18 stationary phase. Amixture of acetonitrile and Water in a proportion of 18:82 v/v wasproved to be the most suitable of all combinations since the chromatographic peaks werebetter defined and resolved and almost free from tailing.The retention time obtained forANA was 3.766 min.Each of the samples was injected threee times and the reproducible retention times wereobserved in all cases.

System suitability parameters were studied with six replicates standard solution ofthe drug and the calculated parameters are within the acceptance criteria. The tailingfactor and the number theoretical plates are in the acceptable limits. The peak areas of ANA were reproducible as indicated by lowcoefficient of variation. A good linear relationship (r=0.998) was observed between theconcentration of ANAand the respective peak areas. The regression curve wasconstructed by linear regression fitting and its mathematical expression was Y= 163.1x + 98.90 (where Y gives peak area and X is the concentration of the drug).

When ANA solutions containing20 , 30 and 28 PPM was analysed by the proposed method for finding out intra and interdayvariations, low % RSD was observed. High recovery values obtained from thedosage form by the proposed method indicatedmethod accuracy. The absence ofadditional peaks indicated non-interference of common excipients used in the tablets.The drug content in tablets was quantified using the proposed analytical method.The tablets were found to contain an average of 98-100% of the labeled amount ofthe drug. The deliberate changes in the method have not much affected the peak tailing,theoretical plates and the percent assay. This indicates that the present method is robust.The lowest values of LOD and LOQ was obtained by the proposed method indicate themethod is sensitive. The standard solution of the drug was stable up to 24 hours as thedifference in percent assay is within acceptable limit. Hence the author proposed that the present HPLC method was sensitive and reproducible for the analysisof ANA in pharmaceutical dosage forms with short analysis time

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