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A NEW SIMPLE ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE ESTIMATION OF ANTI-DIABETIC DRUG LINAGLIPTIN IN BULK AND MARKETED PHARMACEUTICAL DOSAGE FORMS BY USING RP-HPLC

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Abstract:

A simple, selective, rapid and precise reversed-phase high-performance liquid chromatographic method for analysis of Linagliptin in bulk and tablet dosage form has been developed and validated. Chromatography was performed on a Waters C18, 5µm particle size, 25cmx4.6mm i.d. with Phosphate buffer (pH adjusted to 5.8 with orthophosphoric acid): Acetonitrile in the ratio of 45:55 v/v as mobile phase at a flow rate of 1.0 mL min−1. UV detection was performed at 243 nm. Total run time was 9.0 min. Linagliptin were eluted with retention time of 4.398 minutes. The method was validated for accuracy, precision, linearity, specificity, and sensitivity in accordance with USP and ICH guidelines. Validation revealed the method is specific, rapid, accurate, precise, reliable, and reproducible. Calibration plots were linear over the concentration ranges 0-50µgmL−1 for Linagliptin. Limit of detection were 0.003mg mL−1 and limit of quantification were 0.009mg mL−1 for Linagliptin. The high recovery and low coefficients of variation confirm the suitability of the method for analysis of the drug in tablet dosage form. The validated method was successfully used for quantitative analysis of Linagliptin in marketed pharmaceutical dosage forms.

Key Words: *Linagliptin, RP-HPLC, Method Development, Accuracy, Precision.*

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INTRODUCTION:

Linagliptin is a DPP-4 inhibitor developed by Boehringer Ingelheim for the treatment of type II diabetes. Linagliptin differs from other DPP-4 inhibitors in that it has a non-linear pharmacokinetic profile, is not primarily eliminated by the renal system, and obeys concentration dependant protein binding[1]. Linagliptin is a potent, orally bioavailable dihydropurinedione-based inhibitor of dipeptidyl peptidase 4 (DPP-4), with hypoglycemic activity. The inhibition of DPP-4 by Linagliptin appears to be longer lasting than that by some other DPP-4 inhibitors tested. Linagliptin is indicated for the treatment of type II diabetes in addition to diet and exercise5. It should not be used to treat type I diabetes or in diabetic ketoacidosis[2]. An extended-release combination product containing Empagliflozin, Linagliptin, and metformin was approved by the FDA in January 2020 for the improvement of glycemic control in adults with type 2 diabetes mellitus when used adjunctively with diet and exercise. The IUPAC Name of Linagliptin is 8-[(3R)-3-aminopiperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methyl quinazolin-2-yl) methyl] purine-2, 6-dione[3]. The Chemical Structure of Linagliptin is shown in following figure-1.

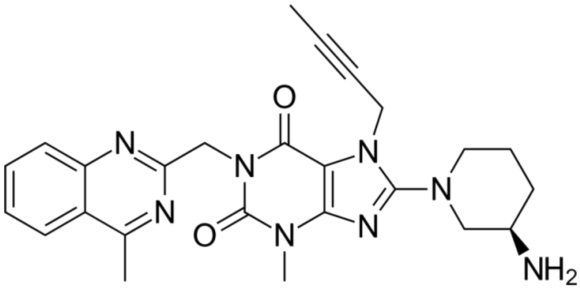

Fig-1: Chemical Structure of Linagliptin

METHOD DEVELOPMENT

1. List of Instruments Used:

Table-1: List of Instruments Used

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector.
2.	**ELICO SL-159** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm
6.	P^H Analyzer **(ELICO)**
7.	Vacuum filtration kit **(BOROSIL)**

2. List of Chemicals, Reagents and Standards Used:

Table-2: List of chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
4.	Potassium dihydrogen orthophosphate	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
5.	Triethyl amine	99.9	L.R.	Sd fine-Chem ltd; Mumbai
6.	Glacial acetic acid	99.99	L.R	Sd fine-Chem ltd; Mumbai

Method Development

HPLC Instrumentation & Conditions: The HPLC system employed was **HPLC** with Empower2 Software with Isocratic with UV-Visible Detector.

Standard & Sample Preparation for UV-Spectrophotometer Analysis:

10 mg of Linagliptin standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 1.0 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase. The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis[4].

It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Linagliptin, so that the same wave number can be utilized in HPLC UV detector for estimating the Linagliptin.

Sample & Standard Preparation for the HPLC Analysis

10 mg of Linagliptin standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with mobile phase.

Further dilution was done by transferring 0.1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Optimization of Chromatographic Conditions: The chromatographic conditions were optimized by different means. (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation etc[5].

Table-3: Summary of Optimization of Process

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	Methanol : Acetonitrile = 60 : 40	1.0 ml/min	243 nm	Broad Peak	Method rejected
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	Methanol : Acetonitrile = 25 : 75	1.0 ml/min	243 nm	Peak broken at the end	Method rejected
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	Acetate Buffer: Acetonitrile = 40 : 60	1.0 ml/min	243nm	Splitting of peak	Method rejected
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	0.1M Phosphate Buffer (P^H 4.0): Acetonitrile = 30 : 70	1.0 ml/min	243 nm	Tailing peak	Method rejected
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	0.1M Phosphate Buffer (P^H 5.2): Acetonitrile = 40 : 60	1.0 ml/min	243nm	Splitting of peak	Method rejected
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	0.1M Phosphate Buffer (P^H 5.8): Acetonitrile = 45 : 55	1.0 ml/min	243 nm	Good Peak	Method accepted

Method Validation:

HPLC method validation establishes documented evidence that an analytical procedure is reliable, accurate, and suitable for its intended purpose (e.g., QC testing, stability studies) according to ICH Q2 (R1) guidelines[11,12,25,30]. Key parameters for publication include specificity, linearity, precision (repeatability/intermediate), accuracy, LOD/LOQ, robustness, and system suitability.

Method Validation Parameters:

Specificity: The ability to assess the analyte unequivocally in the presence of components like impurities, degradation products, or matrix components[6].

Linearity: The ability to obtain test results directly proportional to the concentration of the analyte within a given range, generally with a correlation coefficient (r2)

Accuracy: The closeness of agreement between the true value and the value found, typically determined by % recovery (e.g., spike studies).

Precision: The closeness of agreement between a series of measurements[7].

Repeatability (Intra-assay): Precision under the same conditions over a short time.

Intermediate Precision: Within-laboratory variations (different days, analysts, or equipment).

Limit of Detection (LOD): The lowest amount of analyte that can be detected, often estimated using a signal-to-noise ratio of 3:1.

Limit of Quantitation (LOQ): The lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy, often 10:1 S/N ratio[8].

Range: The interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity.

Robustness: A measure of the method's capacity to remain unaffected by small, deliberate variations in method parameters (e.g., flow rate, pH, temperature).

System Suitability: A check to ensure the HPLC system is working properly before testing, defined by parameters like resolution, theoretical plates and tailing factor[9].

RESULTS AND DISCUSSION:

Analytical Method Development:

Standard & Sample Preparation for UV-Spectrophotometer Analysis:

It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Linagliptin, so that the same wave number can be utilized in HPLC UV detector for estimating the Linagliptin.

While scanning the Linagliptin solution we observed the maxima at 243nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450. The scanned UV spectrum is attached in the following page

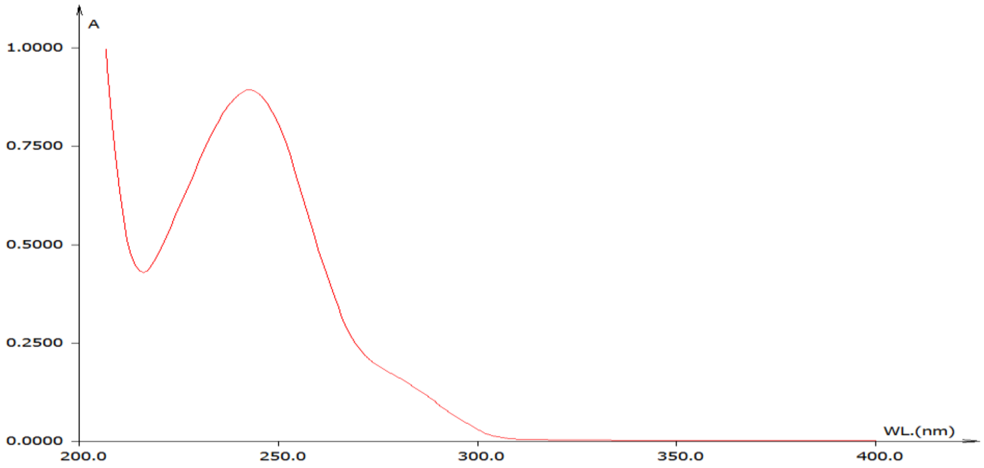

Fig-2: UV Spectrum for Linagliptin (243nm)

Summary of Optimized Chromatographic Conditions:

The Optimum conditions obtained from experiments can be summarized as below[10]:

Table-4: Summary of Optimised Chromatographic Conditions

Mobile phase	0.01M Phosphate Buffer(P^H 5.2): Acetonitrile = 45 : 65
Column	Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm
Column Temperature	Ambient
Detection Wavelength	243 nm
Flow rate	1.0 ml/ min.
Run time	09 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic
Retention time	4.398 minutes

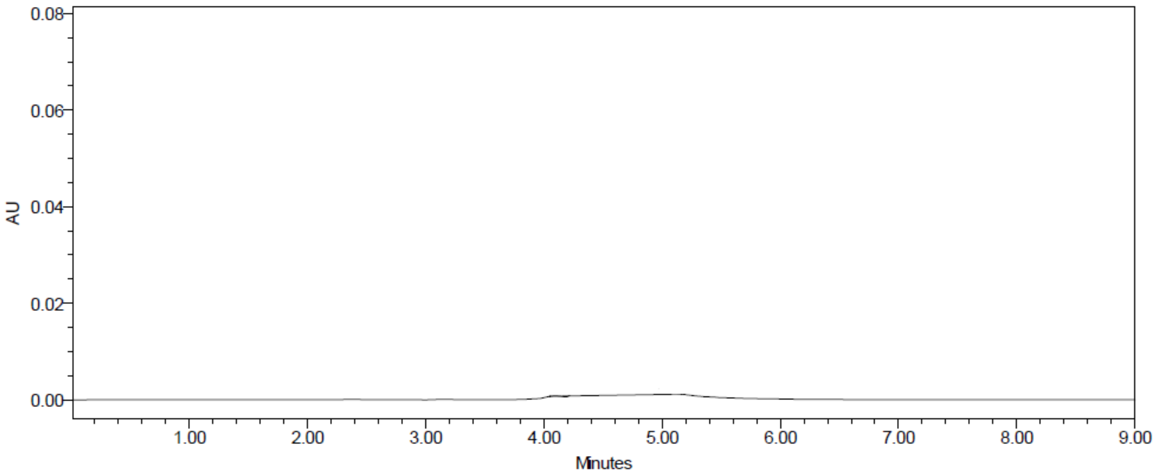

Fig-3: HPLC Spectrum of Linagliptin (Blank Solution)

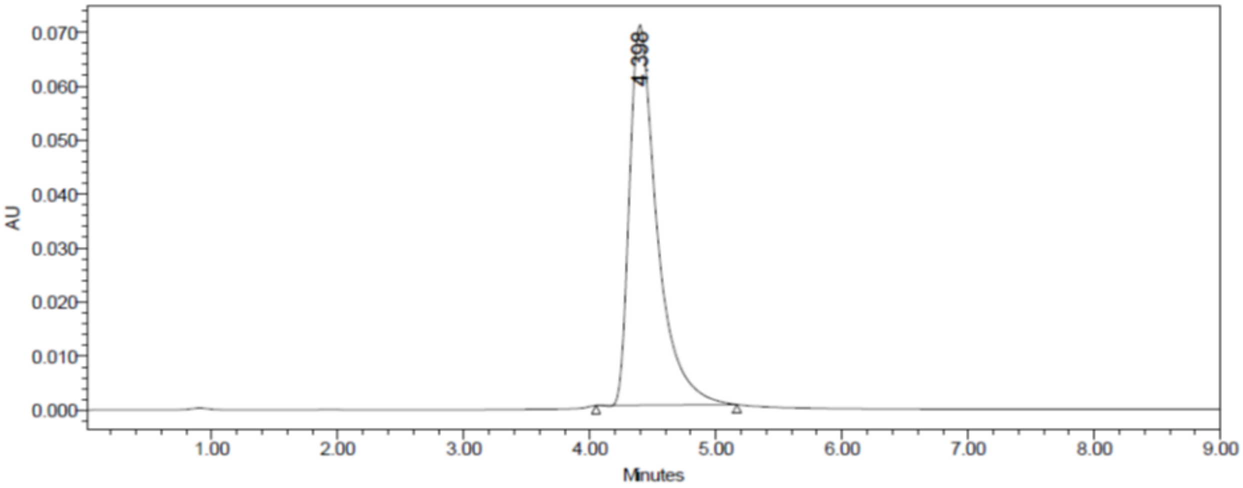

Fig-4: Chromatogram of Linagliptin in Optimized Chromatographic Condition

Validation of Analytical Method:

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application[13]. The RP-HPLC method developed was validated according to International Conference on Harmonization guidelines for validation of analytical procedures[11,12,25,30]. The method was validate for the parameters in terms of system suitability, selectivity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantitation (LOQ).

1. Accuracy:

Recovery Study: To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Linagliptin were taken and added to the pre-analyzed formulation of concentration 50µg/ml. From that percentage recovery values were calculated[14]. The results were shown in Table-5.

Table-5: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	12	12.00308	598252	100.0256	Mean= 99.53621% S.D. = 0.82746 % R.S.D.=0.831315
S_2 : 80 %	12	11.8297	589612	98.58084	
S_3 : 80 %	12	12.00027	598112	100.0022	
S_4 : 100 %	15	15.06891	751035	100.4594	Mean= 99.8473%

S_5 : 100 %	15	14.93017	744121	99.5345	S.D. = 0.530134 % R.S.D.= 0.530944
S_6 : 100 %	15	14.9322	744222	99.54801	
S_7 : 120 %	18	18.02445	898321	100.1358	Mean= 99.51092% S.D. = 1.233106 % R.S.D.= 1.239166
S_8 : 120 %	18	17.65629	879974	98.09047	
S_9 : 120 %	18	18.05517	899852	100.3065	

2. Precision:

Repeatability: The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug[15-17]. Linagliptin (API) the percent relative standard deviations were calculated for Linagliptin is presented in the Table-6.

Table-6: Repeatability Results of Precision

HPLC Injection Replicates of Linagliptin	Retention Time	Peak Area
Replicate – 1	4.399	9677961
Replicate – 2	4.399	9739160
Replicate – 3	4.398	9673810
Replicate – 4	4.392	9649981
Replicate – 5	4.392	9606331
Replicate – 6	4.393	9616451
Average		9660616
Standard Deviation		48279.61
% RSD		**0.49975**

Intermediate Precision:

Intra-Assay & Inter-Assay: The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Linagliptin revealed that the proposed method is precise[18-20].

Table-7: Results of Intra-Assay & Inter-Assay

Conc. of Linagliptin (API) (µg/ml)	Observed Conc. of Linagliptin (µg/ml) by the Proposed Method			
	Intra day		Inter day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
80	79.98	0.99	81.99	1.05
100	100.99	0.98	99.99	0.96
120	119.98	1.02	118.98	0.95

3. Linearity & Range: The calibration curve showed good linearity in the range of 0-50µg/ml, for Linagliptin (API) with correlation coefficient (r^2) of 0.999 (Fig-5). A typical calibration curve has the regression equation of y = 49834x - 9069 for Linagliptin.

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 10, 20, 30, 40, 50 and 70µg/ml. The prepared solutions were filtered through Whatmann filter paper (No.41). From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis). The results which are given in Table below were within acceptable limits[21-23].

Acceptance criteria: Correlation Coefficient should not be less than 0.990.

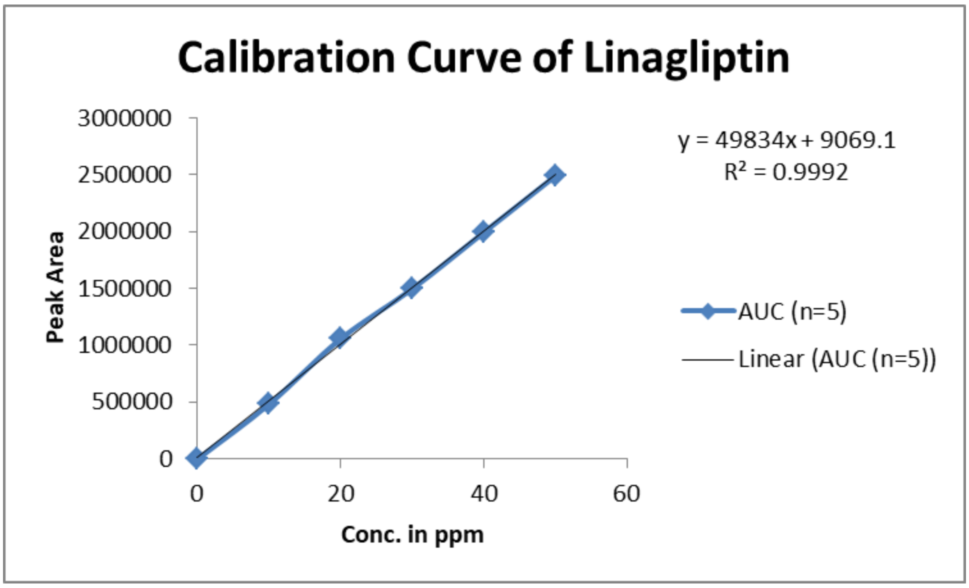

Fig-5: Calibration Curve of Linagliptin (API)

Table-8: Linearity Results of Linagliptin

CONC.	AUC (n=5)
0	0
10	483629
20	1058421
30	1498715
40	1995632
50	2493121

Method Robustness: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & Acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-9, % RSD<2%) the developed RP-HPLC method for the analysis of Linagliptin (API)[24-25].

Table-9: Results of Method Robustness

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.08
Flow (0.9 ml/min)	0.67
Temperature (27^{0}C)	0.75
Temperature (23^{0}C)	0.58
Wavelength of Detection (285 nm)	0.63
Wavelength of detection (281 nm)	0.82

LOD & LOQ: The LOD and LOQ were calculated by the use of the equations

$$LOD = 3.3 \times \sigma / S \text{ and } LOQ = 10 \times \sigma / S$$

Where,

σ is the standard deviation of intercept of Calibration plot and S is the average of the slope of the corresponding Calibration plot[26].

Conclusion: The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.003 & 0.009 µg/ml respectively.

*Y = bx + a where x is the concentration Linagliptin in µg / ml and Y is the Absorbance at the respective λmax.

Assay of Linagliptin in Pharmaceutical Dosage Form:

Twenty tablets/Capsules were taken and the I.P. technique was taken after to decide the normal weight. Above measured tablets were at long last powdered and triturated well. An amount of powder proportionate to 100 mg of medications were exchanged to 100 ml volumetric flagon, and 70 ml of HPLC review methanol was included and arrangement was sonicated for 15 minutes, there after volume was made up to 100 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with HPLC review methanol. The arrangement was separated through a film channel (0.45µm) and sonicated to degas. From this stock arrangement (3.5 ml) was exchanged to five distinctive 10 ml volumetric flagons and volume was made up to 10 ml with same dissolvable framework[27].

The arrangement arranged was infused in five reproduces into the HPLC framework and the perceptions were recorded.

A copy infusion of the standard arrangement was additionally infused into the HPLC framework and the pinnacle zones were recorded. The information is appeared in Table-44.

ASSAY:

$$\% \text{ Assay} = \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{\text{DT}}{\text{WT}} \times \frac{\text{P}}{100} \times \frac{\text{AW}}{\text{LC}} \times 100$$

Where:

AT = Peak Area of Test obtained with test preparation
AS = Peak Area of Standard obtained with standard preparation
WS = Weight of working standard taken in mg
WT = Weight of sample taken in mg
DS = Dilution of Standard solution
DT = Dilution of sample solution
P = Percentage purity of working standard

Assay was performed as described in previous chapter[28]. Results obtained are tabulated below:

Table-10: Assay of Linagliptin Tablets

Brand Name of Tablets	Labelled Amount of Drug (Mg)	Mean (±SD) Amount (Mg) Found By The Proposed Method (N=6)	% Purity
Ondero 5mg Tablet (Lupin Ltd)	5mg	4.547 (± 0.369)	99.785% (± 0.654)

Observation: The amount of drug in Ondero 5mg Tablet was found to be 4.547 (± 0.369) mg/tab for Linagliptin and 99.785 (± 0.654) mg/tab for Linagliptin.

Forced Degradation Studies

The results of the stress studies indicated the **Specificity** of the method that has been developed. Linagliptin was stable in both oxidation & acidic stress conditions[29-30]. The result of forced degradation studies are given in the following Table-11.

Table-11: Results of Forced Degradation Studies of Linagliptin API.

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	90.21	15.29	100.0
Basic Hydrolysis (0.1M NaOH)	24Hrs.	89.28	20.72	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	90.31	24.69	100.0
UV (254nm)	24Hrs.	91.33	8.67	100.0
3 % Hydrogen peroxide	24Hrs.	93.02	6.98	100.0

SUMMARY AND CONCLUSION:

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Linagliptin, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, phosphate buffer, water, 0.1N NaOH, 0.1NHCl). Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Linagliptin it is evident that most of the HPLC work can be accomplished in the wavelength range of 210-300 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 10 µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay and stability related impurity studies which can help in the analysis of Linagliptin in different formulations.

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