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IMPURITY PROFILING STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION OF LINAGLIPTIN AND LC-MS CHARACTERIZATION OF OXIDATIVE DEGRADATION PRODUCT

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ABSTRACT
In this research study, we developed a new rapid and simple HPLC quantitative approach for comprehensively measuring related substance impurities (LNGN N formyl; LNGN acetamide; LNGN N Boc; LNGN amino) in Linagliptin. The related substance impurities were studied by PDA detection at 225 nm after being separated on the Kromasil C18 column stationary phase. The isocratic mode elution and a flow velocity of 1.0 mL/min were performed with the 0.1% phosphoric acid having pH 2.5 and acetonitrile. The proposed approach was appropriately verified in agreement with the "International Conference on Harmonization". Linearity was demonstrated for values ranging from LOQ quantity ppm to 150% quantity ppm, with variance coefficients >0.99. The precision varied from 0.128 to 0.969% RSD and accuracy varied from 99.13% to 101.76% for four related substance impurities. The proposed approach was appropriately implemented in the quality control analysis of the linagliptin API. Stress testing on the linagliptin API is also part of this investigation. One degradant with a 10.273 min Rt was found during oxidative stress testing on linagliptin. Using LC-MS this peak was discovered to be an adduct.
Keywords: Related Substances, Impurities, Quality Control, Linagliptin.
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INTRODUCTION

The incidence along with the overall occurrence of type 2 diabetes mellitus is quite high worldwide.[1] Incretin hormones are dysfunctional in people experiencing type two diabetes. It is feasible to decrease the incretin hormone inactivation and promote blood glucose reductions in a glucose-dependent approach by suppressing the dipeptidyl peptidase-four enzyme.[2] Linagliptin (LNPN, Fig.-1) is a very effective as well as a selective antagonist of the dipeptidyl peptidase-four enzyme presently permitted for the therapies of type 2 diabetes.[3,4] "Related substances (ReS)" refers to all conceivable chemicals that an API may degrade into or be constructed from. All process impurities, intermediates, plus degradants are included in this. The safety, effectiveness, and sustainability of pharmaceutical formulation preparations and APIs are correlated with ReS.[5,6] As a necessary consequence, ReS and degradation components in various drugs and the final pharmacological formulations were the subjects of relatively high regulatory and pharmacopeia attention.[7] Few investigation publications have been posted online in the past studies on the assessment of LNPN-related impurities in pharmaceutical batches, including tablets (RP-HPLC[8]) and API batches (UHPLC,[9] HILIC,[10,11] HPLC[12,] and LC-PDA[13]). In this, we investigated four ReS (Fig.-1) of LNGN. Nevertheless, no researchers have yet been able to make use of any organized investigation into the quality control of the four ReS of LNGN under investigation. Hence, the need for a quick, easy, and stable technique to determine the four ReS of LNGN under investigation is important. For the assessment of four ReS of LNGN, a rapid and simple HPLC quantitative approach was established in this research work.

EXPERIMENTAL

HPLC Equipment and Conditions

The Waters 2965 HPLC apparatus served to record the HPLC analyses of LNGN and its four ReS employing Kromasil C18 immobile column (250 mm: 5 µm: 4.6 mm) in a thermostatic column warmer at 30 °C. The isocratic mode (65% volume 'A':35% volume 'B') elution and a stream velocity of 1.0 mL/min

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were performed with the mobile phase "A" (i.e., 0.1% phosphoric acid having pH 2.5, Merck Ltd, India) and "B" (i.e., acetonitrile, Merck Ltd, India). The LNGN and its four ReS at 225 nm were detected using a PDA (Waters, US) and this information was utilized to evaluate them. The diluent for LNGN and its four ReS sample preparations was a perfect blend of water (milli-Q) plus acetonitrile that was mixed 50% volume to 50% volume. Every HPLC analysis of LNGN and its four ReS was performed using just 10 µl sample injections.

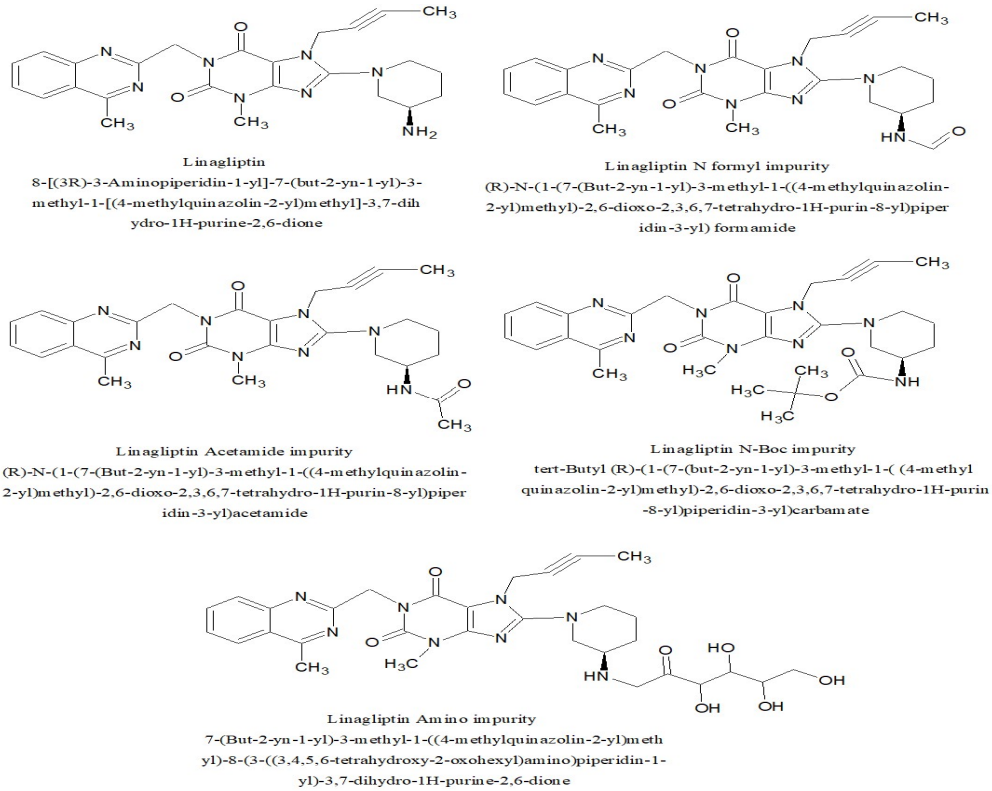


Fig.-1: Chemical Information: LNGN and its Four ReS

LNGN Solutions

Olympus Chemicals and Fertilizers, India supplied the test LNGN substances as a free sample. To obtain the stock LNGN solution (5000 ppm), 50.0 mg of standard LNGN material was balanced and dissolved utilizing sonication over five min in 10 mL of diluent. Thereafter, a working LNGN solution with a content of 500 ppm LNGN was generated by diluting 1000 µl of the prepared stock LNGN solution (5000 ppm) to 10 ml employing diluent.

ReS Solutions

Olympus Chemicals and Fertilizers, India supplied the opted four ReS substances as a free sample. For the stock ReS solution (3000 ppm), 10 ml of diluent served to dissolve 30.0 mg of each standard ReS substance individually. Thereafter, a working ReS solution with a content of 300 ppm each ReS was generated by diluting 1000 µl of the prepared stock ReS solution (3000 ppm) to 10 ml employing diluent. Using diluent at the following concentrations, working ReS solutions for calibration curve generation were created: 0.463 ppm to 450 ppm (LNGN N formyl impurity), 0.574 ppm to 450 ppm (LNGN acetamide impurity), 0.045 ppm to 450 ppm (LNGN N Boc impurity), 0.115 to 450 ppm (LNGN amino impurity).

Calibration Curves of Opted Four ReS

Seven solutions of a blend of ReS substances are employed to generate ReS substance calibration curves. The ReS substances calibration curves were accomplished at ranges between: 0.463 ppm to 450 ppm (LNGN N formyl impurity), 0.574 ppm to 450 ppm (LNGN acetamide impurity), 0.045 ppm to 450 ppm (LNGN N Boc impurity), 0.115 to 450 ppm (LNGN amino impurity). Through the application of the least-square approach, the calibration curve, which depicts the linear connection between the ReS solution concentration and the peak area, was created.

Analysis of Opted Four ReS Analysis in LNGN Sample

This was accomplished by quantifying four opted ReS substances in test LNGN API samples. Under ideal chromatographic circumstances, the sample LNGN API solutions are made and evaluated. The peak areas of the four specified ReS substances and their accompanying calibration graphs were assessed to identify the amounts of the four specified ReS substances in LNGN.

LNGN Degradation Study

Following ICH norms and recommendations, an investigation on LNGN degradation was conducted.[14]

Acid/Base/Peroxide-Based Hydrolysis

Three 10 ml volumetric flasks were filled with a specified volume (1 mL) of the working LNGN solution instead of stress experiments. The following step involved adding a specified volume (1 mL) of 1 M HCl (Rankem India Ltd), 1 M NaOH (Rankem India Ltd), or 30% peroxide (Rankem India Ltd) into 3 flasks for acid, or alkali, or peroxide hydrolysis, respectively. The three flasks were refluxed at the lab temperature over 24 hr before being brought up to the correct level (10 ml) with a diluent. The solutions were subsequently filtered, fed onto the Kromasil C18 immobile column (250 mm: 5 µm: 4.6 mm), and examined using ideal chromatographic circumstances.

Photo/Thermal Based Degradation

Two 10 ml volumetric flasks were filled with a specified volume (1 ml) of the working LNGN solution in place of these stress experiments. In an attempt to demonstrate LNGN photodegradation, one flask was housed in an ultraviolet chamber (Ultra-Lum, USA) for seven days. To examine LNGN for thermal degradation, the other flask was warmed in an oven (Serve Well Instrument Ltd, India) for 24 hr around 120 °C. The volume in flasks is brought up to the correct level (10 ml) with a diluent. The solutions were subsequently filtered, fed onto the Kromasil C18 immobile column (250 mm: 5 µm: 4.6 mm), and examined using ideal chromatographic circumstances.

LC-MS Conditions

The MS execution took place in a "positive ion electrospray ionization" interface manner. Other MS settings that were customized included: temperatures: Drying gas - 120-250°C, Source - 550ºC; Voltage: Ion spray - 5500 V, Collision drive - 15 V; Collision gas: nitrogen; Potential: Entrance - 10V, Declustering - 40 V, Exit - 7 V; Drying gas flow stream: 5 L/min; and Dwell period: 1sec

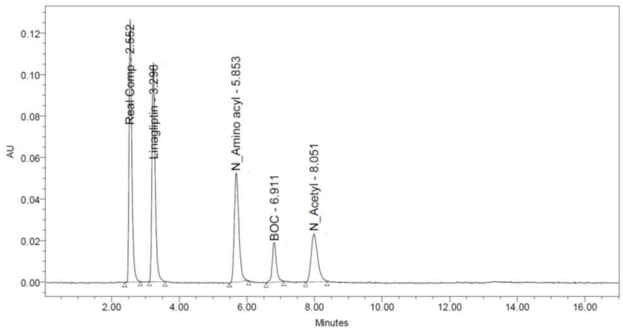
RESULTS AND DISCUSSION

Establishment of Ideal Chromatographic Circumstances for Opted Four Res Analysis

The time needed for the four ReS assessments, the repeatability of the four ReS persistence durations, the segregation of the four ReS peaks, and the fineness, resolution, as well as symmetry of the four ReS peaks, served as the decisive factors for establishing the highest quality HPLC settings. For overall finer separation as well as quantification of the specified four ReS peaks, three distinctive C18 columns (Ascentis - 2.8 µm, 4.8 mm, 150 mm; Inertisil - 4.8 µm: 4.6 mm: 250 mm; and Kromasil - 5.0 µm: 4.6 mm: 250 mm) were explored as stationary phases. The Kromasil (5.0 µm: 4.6 mm: 250 mm) column was adopted in this investigation because it displayed improved separation properties for specified four ReS peaks. Acetonitrile-phosphoric acid (0.1% aqueous, possess pH 2.5) and methanol-phosphoric acid (0.1% aqueous, possess, pH 2.5), at various organic to aqueous solvent proportions, are the multiple mobile phases that were researched for enhanced peak resolutions of ReS as well as separation of ReS. Nevertheless, following several experimental tests, it was revealed that the requisite four ReS could be best separated using an isocratic solvent combination (35% volume and 65% volume) of acetonitrile - phosphoric acid (0.1% aqueous, possessing pH 2.5). The ideal UV wavelength was 220 nm, the ideal flow rate seemed to be 1.0 mL/min, the ideal column warmth remained at 30 °C, and an ideal injecting level seemed to be 10 µl. A typical ReS sample solution's HPLC chromatogram is illustrated in Fig.-2 under ideal chromatographic circumstances.

Validation

Following ICH norms and recommendations, an investigation on the developed method's validation was conducted.[15]

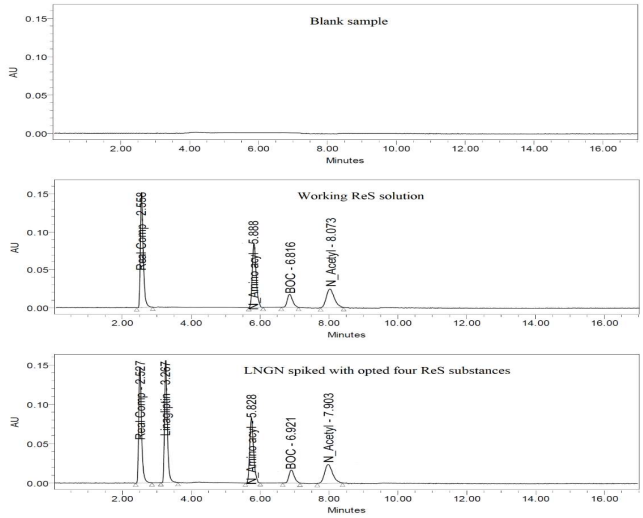


In figure: Real comp – LNGN N formyl impurity; Linagliptin – LNGN; N_Amino acyl – LNGN amino impurity; Boc – LNGN N-Boc impurity; and N_ Acetyl – LNGN acetamide impurity.

Fig.-2: A Typical Res Sample Solution's HPLC Chromatogram

Selectivity

This parameter was assessed to find any interference in the chromatographic separation of the four specified ReS substances with a blank sample (blend of water (milli-Q) plus acetonitrile that was mixed 50% volume to 50% volume) and LNGN API sample. There wasn't any interference with both the blank (Fig.-3) and LNGN API (Fig.-3) samples. The approach is proven to analyze four specific ReS substances in LNGN samples.



In figure: Real comp – LNGN N formyl impurity; Linagliptin – LNGN; N_Amino acyl – LNGN amino impurity; Boc – LNGN N-Boc impurity; and N_ Acetyl – LNGN acetamide impurity.

Fig.-3: - Blank, ReS Sample, and Spiked LNGN Solution's HPLC Chromatogram

System Suitability

With exactly the same quantities (300 ppm) of four specified ReS substances injected in six replicates, the method's system adaptability was tested, and the area, resolution, Rt, along tailing factor four specified ReS substances were evaluated. After examining the results of six analysis replications, it was assessed that there weren't are no discernible variations in the responses of four specified ReS substances. The strategy's RSD (%) for system suitability was noticed to be <2 (0.307% to 1.667%), which signified a significant level of instrument precision.

Table-1: System Suitability for Four Specified ReS Substances Evaluation

	Peak area	Plate count	Tailing	Resolution
LNGN N formyl impurity				
Mean attained*	910602	5782	1.394	-
SD (±)	2795.880	90.388	0.011	-
RSD (%)	0.307	1.563	0.818	-
LNGN acetamide impurity				

Mean attained*	384904	7227	1.270	5.020
SD (±)	2810.102	52.545	0.010	0.084
RSD (%)	0.730	0.727	0.787	1.667
LNGN N Boc impurity				
Mean attained*	166836	14338	1.206	3.360
SD (±)	264.833	331.591	0.015	0.055
RSD (%)	0.159	1.313	1.258	1.630
LNGN amino impurity				
Mean attained*	617469	8477	1.328	8.940
SD (±)	7356.543	97.557	0.008	0.055
RSD (%)	1.191	1.151	0.630	0.613

* Average attained from five values; SD – standard deviation attained for five values;
RSD – relative standard deviation

Sensitivity

Depending upon signal comeback-to-noise response proportions of 3:1 (for LOD) as well as 10:1 (for LOQ), the LODs, as well as LOQs of four specified ReS substances for the proposed approach, were calculated (LOQ). The LODs were 0.153 ppm for LNGN N formyl impurity, 0.038 ppm for LNGN amino impurity, 0.015 ppm for LNGN N-Boc impurity, and 0.190 ppm for LNGN acetamide impurity. The LOQs were 0.463 ppm for LNGN N formyl impurity, 0.115 ppm for LNGN amino impurity, 0.045 ppm for LNGN N-Boc impurity, and 0.574 ppm for LNGN acetamide impurity. The lowest detectable (LOD) and lowest quantifiable (LOQ) concentrations of four specified ReS substances proved sensitivity.

Linearity

Working ReS solutions for calibration curve generation were created and analyzed. The detector responses of four specified ReS to different concentrations [0.463 ppm to 450 ppm (LNGN N formyl impurity), 0.574 ppm to 450 ppm (LNGN acetamide impurity), 0.045 ppm to 450 ppm (LNGN N Boc impurity), 0.115 to 450 ppm (LNGN amino impurity)] resulted in a linear association with following regression equations:

$y = 3030x + 6185.6$; $R^2 = 0.9999$ for LNGN N formyl impurity
$y = 1981.2x + 5874.6$; $R^2 = 0.9999$ for LNGN amino impurity
$y = 559.39x + 1239$; $R^2 = 0.9998$ for LNGN N Boc impurity
$y = 1276.5x + 2744.2$; $R^2 = 0.9998$ for LNGN acetamide impurity

For the concentration ranges of four specified ReS investigated, the regression plots (Fig.-4) revealed a virtually satisfying linear relationship (variance coefficients were >0.99).

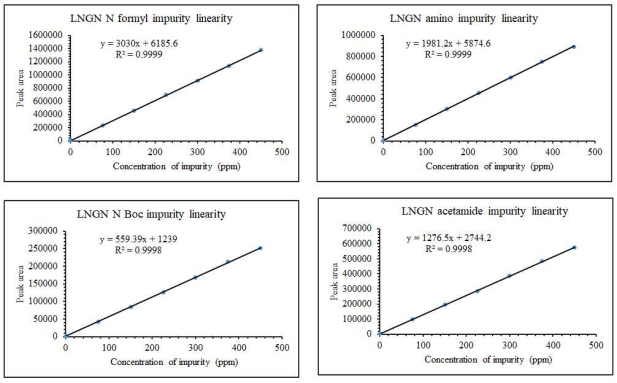


Fig.-4: Linearity Graphs of Four Specified ReS

Method Precision

An examination of repeatability was used to gauge the precision. The precision was predicted by dint of assessing five duplicates of each LNGN API sample having added concentrations equivalent to 300 ppm of LNGN N formyl impurity, LNGN amino impurity, LNGN N-Boc impurity, and LNGN acetamide impurity. The RSD, which symbolized the repeatability of the findings, served to compute precision. The RSD varied from 0.128 to 0.969% for four specific ReS substances' peak areas. Table-2 summarizes the findings. Considering the data obtained, the current approach displayed noticeably better repeatability.

Table-2: Precision for Four Specific ReS Substances

Sample No.	Impurity peak areas			
	LNGN N formyl	LNGN amino	LNGN N-Boc	LNGN acetamide
1	917581	613279	166817	381293
2	912490	614038	169897	380517
3	916349	615134	165999	380177
4	911662	613872	169195	380177
5	910760	619769	167529	380932
Mean	913768	615218	167887	380619
SD (RSD)	3013.184 (0.330)	2630.708 (0.428)	1627.004 (0.969)	488.205 (0.128)

Accuracy

The accuracy was evaluated via a recovery analysis. The accuracy was predicted by assessing three duplicates of each LNGN API sample having added concentrations equivalent to 50% specification limit contents, 100% specification limit contents, and 150% specification limit contents of LNGN N formyl impurity, LNGN amino impurity, LNGN N-Boc impurity, and LNGN acetamide impurity. Lastly, the concentrations of four specific ReS substances from the detection findings were computed to determine the spiking recoveries of four specific ReS substances (Table-3). The measured recoveries of four specific ReS substances in this study varied from 99.13% to 101.35% for LNGN N formyl impurity, 99.14% to 99.96% for LNGN amino impurity, 100.83% to 101.61% for LNGN N Boc impurity and 100.37% to 101.76% for LNGN acetamide impurity, showed considerably stronger accuracy.

Table-3: Accuracy for Four Specific ReS Substances

Accuracy level↓	-	Impurities accuracy			
		LNGN N formyl	LNGN amino	LNGN N-Boc	LNGN acetamide
50% specification level	Added (ppm)	150	150	150	150
	Found (ppm)*	152.02	149.39	152.42	152.63
	Recovery (%)*	101.35	99.96	101.61	101.76
100% specification level	Added (ppm)	300	300	300	300
	Found (ppm)*	301.55	297.42	302.49	301.12
	Recovery (%)*	100.52	99.14	100.83	100.37
150% specification level	Added (ppm)	450	450	450	450
	Found (ppm)*	446.12	446.88	454.57	436.86
	Recovery (%)*	99.13	99.32	101.02	100.81

* mean calculated for three found/recovery values

Degradation Studies

According to ICH, accelerated stress conditions of various kinds [Photo/Thermal degradation and Acid/Base/Peroxide hydrolysis] were applied to the LNGN API. The samples were analyzed for related substances and assay of API. We found higher level degradation (Table-4) in base-based hydrolysis and less significant degradation (Table-3) observed under photo-based degradation. None of the LNGN-related impurities specified in this study (LNGN N formyl impurity, LNGN amino impurity, LNGN N Boc impurity, and LNGN acetamide impurity) were observed in accelerated stress conditions of various kinds. Degradation chromatograms obtained in accelerated stress conditions of various kinds were used to

demonstrate this. None of the chromatograms from Photo/Thermal based degradation or Acid/Base/Peroxide based hydrolysis conditions showed peak relevant to LNGN N formyl impurity/LNGN amino impurity/LNGN N Boc impurity/LNGN acetamide impurity. However, in samples of LNGN API pursuant to oxidative stress, one degradant with 10.273 min Rt was found. The method's specificity, as shown by the peak purity (purity angle and threshold values) for LNGN under various types of accelerated stress applications was also examined. The peak purities for LNGN were passed (purity angles < threshold values, Table-4) and LNGN retention was interference-free.

Table-4: Accelerated Stress Conditions Studied on LNGN API

Degradation condition	LNGN degraded (%)	LNGN assay (%)	RTs of degradants	Peak purity angle	Peak threshold
Acid (1M HCl)	7.16	92.84	-	6.31	8.23
Base (1M NaOH)	8.65	91.35	-	13.74	28.39
Peroxide (30% H_2O_2)	4.51	95.49	10.27	10.91	31.15
Thermal (120 °C)	1.07	98.93	-	15.94	30.81
Photo (UV chamber)	0.69	99.31	-	10.45	16.65

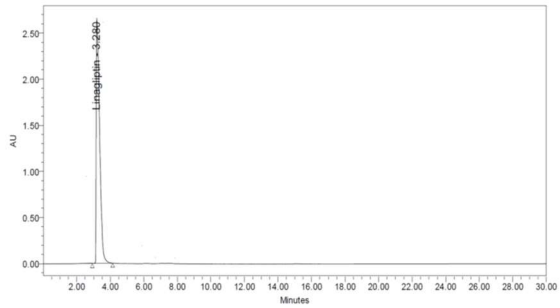


Fig.-5: Chromatogram LNGN After Acid (1M Hcl) Treatment

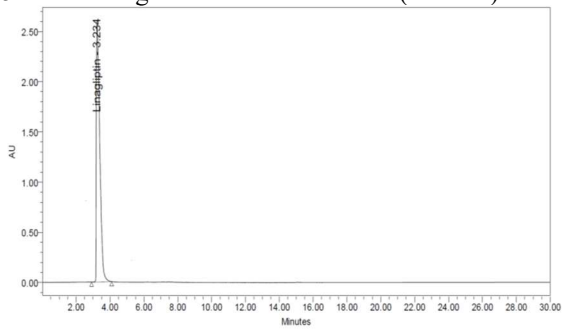


Fig.-6: Chromatogram LNGN After Base (1M Naoh) Treatment

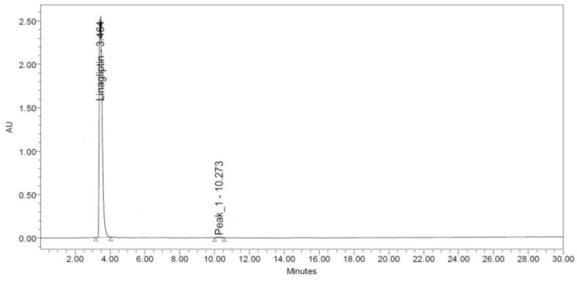


Fig.-7: Chromatogram LNGN after Oxidation (30% Peroxide) Treatment

Characterization of Degradant in Samples of LNGN API Under Oxidative Stress

Utilizing LC-MS (see segment: LC-MS conditions) the detected degradant impurity peak in the strained LNGN API was identified. The SCIEX program was deployed to analyze the detected degradant impurity peak and identify the degradation compound. The computed mass of the degradant impurity peak was 468.51. The most prevalent ion was found to have a m/z ratio of 468.51 in the mass spectrum of the

degradant impurity peak that was discovered (Fig.-10). This peak was generated via a salt reaction in which water and linagliptin were trapped as an adduct, leading to the peak at 468.51.

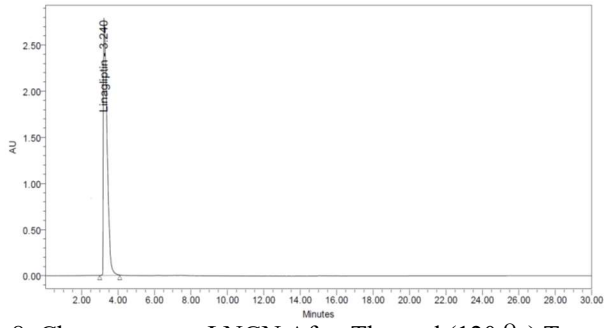


Fig.-8: Chromatogram LNGN After Thermal (120 ^{O}c) Treatment

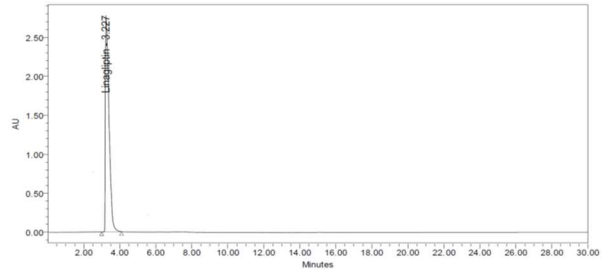


Fig.-9: Chromatogram LNGN After Photo (UV Chamber) Treatment

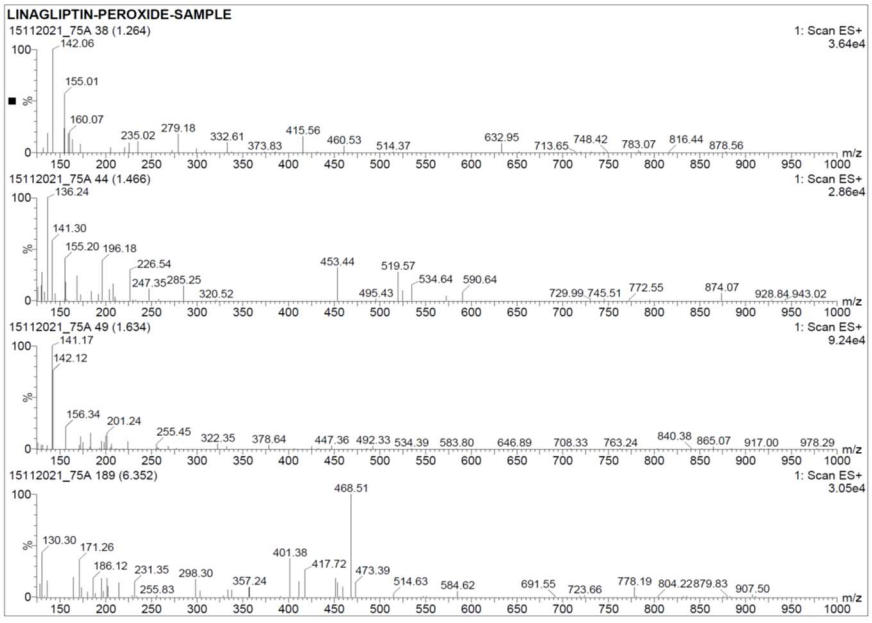


Fig.-10: Mass Spectra of Samples of LNGN API Under Oxidative Stress

Robustness

To comprehend the impact of minor modifications on the experimental situations, the robustness of the newly suggested analytical technique must be inspected in alignment with ICH proposals. The robustness was predicted by assessing the LNGN API sample having added concentrations equivalent to 300 ppm of LNGN N formyl impurity, LNGN amino impurity, LNGN N-Boc impurity, and LNGN acetamide impurity. The flow stream speed (0.9/1.0/1.1 ml/min), the temperature (28°C/ 30°C/ 32°C), and the mobile phase constitution (30%/35%/40%; the volume of acetonitrile was altered evenly to achieve the total quantity 100%) were the factors taken into consideration to study their impact on recovery percentile of four specific ReS substances (Table-5). Small RSD values suggest that a modest variation in any one aspect did not have an impact on the recovery percentile of four specific ReS substances.

Table-5: Robustness for Four Specific ReS Substances

Parameter changed	Found (ppm)*	Recovery (%)	Mean/ RSD	Found (ppm)*	Recovery (%)	Mean/ RSD
	LNGN N formyl impurity			LNGN amino impurity		
Flow stream speed minus (0.9 ml/min)	299.61	99.87	100.023 /0.200	299.85	99.95	99.83/ 0.148
Flow stream speed plus (1.1 ml/min)	299.85	99.95		299.01	99.67	
Optimized flow (1.0 ml/min)	300.75	100.25		299.67	99.89	
MP* minus (30 %)	301.44	100.48	100.38/ 0.081	296.67	98.89	98.76/ 0.122
MP* plus (40%)	300.99	100.33		296.25	98.75	
Optimized MP* (35%)	301.05	100.35		295.95	98.65	
Temp. minus (28 °C)	300.75	100.25	100.63/ 0.334	301.29	100.43	100.47/ 0.155
Temp. plus (32 °C)	302.67	100.89		301.95	100.65	
Optimized Temp. (30 °C)	302.25	100.75		301.05	100.35	
	LNGN N-Boc impurity			LNGN acetamide impurity		
Flow stream speed minus (0.9 ml/min)	301.17	100.39	100.44/ 0.128	304.44	101.48	101.52/ 0.052
Flow stream speed plus (1.1 ml/min)	301.77	100.59		304.74	101.58	
Optimized flow (1.0 ml/min)	301.05	100.35		304.50	101.50	
MP* minus (30 %)	301.29	100.43	100.48/ 0.047	303.75	101.25	100.96/ 0.282
MP* plus (40%)	301.56	100.52		302.04	100.68	
Optimized MP* (35%)	301.50	100.50		302.85	100.95	
Temp. minus (28 °C)	303.75	101.25	101.35/ 0.099	301.89	100.63	100.65/ 0.025
Temp. plus (32 °C)	304.08	101.36		302.04	100.68	
Optimized Temp. (30 °C)	304.35	101.45		301.95	100.65	

* Percent volume of acetonitrile

CONCLUSION

A rapid and simple HPLC quantitative approach was established in this research work for the assessment of four ReS (LNGN N formyl impurity; LNGN acetamide impurity; LNGN N Boc impurity; LNGN amino impurity) of LNGN. According to ICH rules, the established technique was successfully verified to fulfill the specification criteria for every validation parameter. The approach yields extremely specific and reliable findings when used to examine the stability of the LNGN API. The analysis did not involve the use of any potentially harmful chemicals, and the approach uses little volumes of organic solvents, making it both secure and environmentally harmless. During the commercial manufacture of LNGN batches, the established approach may be utilized with effectiveness for routine testing of four ReS (LNGN N formyl impurity; LNGN acetamide impurity; LNGN N Boc impurity; LNGN amino impurity) in pharmaceutical LNGN goods. Also, this investigation involves stress tests on LNGN API. Oxidative stress tests of LNGN API revealed one degradant with a 10.273 min Rt. This degradant was characterized using LC-MS and found to be an adduct of LNGN.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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