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ISOLATION, CHARACTERIZATION OF DEGRADATION PRODUCTS OF SITAGLIPTIN AND DEVELOPMENT OF VALIDATED STABILITY-INDICATING HPLC ASSAY METHOD FOR SITAGLIPTIN API AND TABLETS

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Keywords:

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ABSTRACT: The degradation pathway of sitagliptin in bulk and tablet has been investigated during stress study. Major degradation products were isolated in pure form and characterized using mass and NMR spectroscopy. Three previously unreported impurities were found to be, 3-(trifluoromethyl)-6, 7-dihydro[1,2,4]triazolo[4, 3-a]pyrazin-8(5H)-one, (2E)-1-[3-(trifluoromethyl)-5, 6-dihydro[1, 2, 4]triazolo[4, 3-a]pyrazin-7(8H)-yl]-4-(2, 4, 5-trifluorophenyl)but-2-en-1-one and (3E)-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4, 3-a]pyrazin-7(8H)-yl]-4-(2, 4, 5-trifluorophenyl)but-3-en-1-one. Further, a stability-indicating reverse phase HPLC assay method was developed on Poroshell 120 EC-C18 (3X150mm, 2.7µ) column using mobile phase consisting of 5mM ammonium acetate and acetonitrile with gradient elution in presence of spiked degradation products and impurity. The flow rate was 0.5ml/min and detection was at 210nm. The method was found to be linear over 10µg-500µg/ml (r^2>0.999). The method was validated with respect to accuracy, precision, specificity, robustness and found to be stability indicating.

INTRODUCTION: Sitagliptin phosphate monohydrate (Sitagliptin), (3R)-3-amino-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a] pyrazin-7(8H)-yl]-4-2,4,5-trifluorophenyl)butan-1-one phosphate monohydrate (**Fig. 1**) is oral anti-diabetic drug, marketed as Januvia® by Merck & Co. used to treat Type II diabetes mellitus [1, 2]. Sitagliptin, the first new anti-diabetic drug in DPP IV inhibitors class, enhances level of active GLP-1 & other incretins which potentiate insulin secretion.[3, 4]

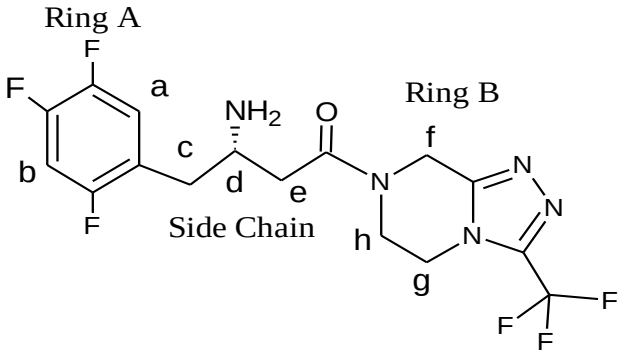

FIG. 1: CHEMICAL STRUCTURE OF SITAGLIPTIN

There have been several methods in the literatures for the determination of sitagliptin alone in bulk and formulation using spectrophotometer [5], in biological fluids using HPLC [6], LC-MS/MS [7], HTLC-MS/MS [8], molecularly imprinted solid-phase extraction (MISPE) combined with Zwitter ionic HILIC [9].

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Some reports described analytical methods in combination with metformin, in biological fluids using laser diode thermal desorption interfaced with APCI-MS/MS [10], another in combination with traditionally used anti-diabetic drugs in aqueous environmental samples using HPLC/Q-TOF MS [11]. Besides, some methods reported for determination of sitagliptin and metformin in presence of sitagliptin's one alkaline degradation product using HPLC [12], and for determination of sitagliptin and metformin in presence of sitagliptin's N-acetyl impurity and metformin related impurities by UPLC [13].

Present work focuses on the study of degradation behavior, isolation and characterization of major degradation products of sitagliptin in bulk and tablet by subjecting to various stress conditions. Further, developed a validated stability indicating mass compatible HPLC method for the determination of sitagliptin in bulk and tablet in presence of its degradation products (DP's) and N-acetylated impurity. Out of the five degradation products reported in this study degradation product namely, DP-II and N-acetylated impurity were known as impurities of sitagliptin and DP-III was reported as an intermediate for the synthesis of sitagliptin [12, 13, 14]. Remaining three degradation products observed during this degradation study (DP-I, DP-IV and DP-V) were found to be novel degradation products of sitagliptin.

EXPERIMENTAL:

Chemicals and Reagents: Sitagliptin phosphate monohydrate salt prepared from the sitagliptin base which was isolated form the Januvia tablet [15, 16]. Januvia® tablets (100mg) were purchased from the local medical store. HPLC grade acetonitrile and methanol were purchased from JT Baker.

Analytical reagents sodium hydroxide, hydrochloric acid, hydrogen peroxide, o-phosphoric acid, ammonium acetate, trifluoro-acetic acid, potassium dihydrogenphosphate, acetic acid, formic acid, triethylamine, dichloromethane, ethylacetate were purchased from Merck.

Synthetic grade acetyl chloride obtained from Spectrochem. HPLC grade water was obtained from in-house Millipore Milli-Q system and used to prepare all solutions.

Instrumentation: Mass spectra were recorded on the Agilent single quadrupole mass spectrometer (LC/MSD VL) with APCI-ESI ionization source. All observed mass values (m/z) presented in this article are given as M^{+1} ions. NMR spectral data were recorded on Varian NMR spectrometer (400MHz) using deuterated solvents CD_3OD, $CDCl_3$, DMSO-d_6 and D_2O obtained from Cambridge Isotope Laboratories, Inc (USA). IR spectra were recorded on PerkinElmer (spectrum 100) FT-IR spectrometer using KBr pellet for solid samples and in DCM on NaCl disc for semisolid sample.

HPLC analysis was carried out using Agilent 1200 series HPLC instrument consisting a degasser, quaternary pump, autosampler with thermostat, column temperature controller, diode array detector and data was processed using ChemStation software. Purifications were carried-out on Agilent 1200 preparative HPLC equipped with prep pump (Binary), auto-sampler, diode array detector and prep fraction collector.

Chromatographic methods and conditions: Initial stress study samples were analyzed on Agilent eclipse XDB C-18 (4.6X250mm, 5µ) column at 25ºC with a flow rate of 1ml/min using mobile phase consisting of 0.1% o-phosphoric acid in water (A) and acetonitrile (B) in gradient elution. The gradient elution program was: 0-3min: 10% solvent B, 3-18min: linear increase to 50% B, 18-30min: linear increase to 100% B, 30-34min 100% B, 34-37min: linear decrease to 5% B, 37-40min 5% B. UV detection were carried out at 210nm and 264nm along with PDA data.

Isolation of degradation products was performed on Agilent preparative system using Agilent PrepHT XDB-C18 (21.2X150mm, 5µ) column and a mobile phase consisting of 5mM ammonium acetate in water and ACN at 20ml/min flow and fractions were collected by monitoring at wavelength 210nm. Final HPLC assay method was developed and validated on Agilent Poroshell 120 EC-C18 (3X150mm, 2.7µ) column using mobile phase 5mM ammonium acetate in water (A) and ACN (B) using gradient elution with a flow rate of 0.5ml/min. The gradient elution program was: 0-1.5min: 8% solvent B, 1.5-6.5min: linear increase to 50% B, 6.5-8.5min: 50% B, 8.5-14min: linear decrease to 15% B, 14-15min: linear decrease to

8% B, 15-18min: 8% B. Column temperature was adjusted to 40ºC and data collected at 210nm wavelength.

Forced degradation of API and Tablets: Stress testing of the drug substance can help identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and to develop and validate the stability indicating power of the procedures used [17]. Stress studies were performed on API and tablet powder using 0.5mg/ml solutions. Considering good solubility of sitagliptin phosphate, all stress study solutions were prepared in water.

Samples were withdrawn from stress study samples at particular intervals and diluted with water: methanol (1:1), in order to achieve solubility of water insoluble impurities and to obtain 0.2mg/ml final concentration. All samples were filtered through 1µ membrane filters before subjecting to LC analysis for monitoring degradation behavior by injecting 15µl of each sample.

Acid degradation studies: Solutions for acid degradation studies were prepared in 0.1N HCl and stored in oven at 55ºC for 2 weeks. Sufficient amount of samples were withdrawn at regular intervals for monitoring degradation of analyte.

Alkali degradation studies: Solutions for alkali degradation studies were prepared in 0.1N NaOH. When stored at 55ºC, rapid degradation was observed, hence, alkali forced degradation study samples were stored at laboratory temperature (25ºC) and sufficient amount of samples were withdrawn at particular intervals for monitoring degradation of analyte.

Neutral degradation studies: Solutions for neutral degradation studies were prepared in water and kept in oven at 55ºC for 2 weeks. Sufficient amount of samples were withdrawn at regular intervals for monitoring degradation of analyte.

Oxidation studies: Solutions for oxidation studies were prepared in 5% H_2O_2 and kept in oven at 55ºC for 2 weeks. Sufficient amount of samples were withdrawn at regular intervals for monitoring degradation of analyte.

Thermal degradation studies in solid state: Thermal studies were performed on solid API and tablet powder samples at 80ºC for 10 days.

Photo stability studies: Drug substance and tablet powder were exposed to light, as a layer (1mm thickness), spread in a petridish using photo stability chamber (Binder, KBF 720-ICH-LIGHT) for 10 days. Following removal of the samples from the light cabinet, 0.2mg/ml conc. samples were prepared for HPLC analysis using methanol: water (1:1) as a diluent.

Isolation of degradation products (DP's) and synthesis of N-acetylated impurity: Based on the degradation profile obtained from forced degradation study, degradation products were generated by subjecting approximately 200mg sitagliptin API to suitable stress condition. The degradation was monitored using HPLC for reasonable conversion in to desired impurities. DP-I was generated by subjecting API to oxidative stress condition using 10% H_2O_2 at 60ºC for four days. DP-II and III were produced by subjecting API to 0.1N NaOH at 60ºC for two days. NaOH degradation sample was neutralized by dil. HCl.

DP-IV and V were generated by refluxing drug tablet powder in water at 130ºC for four days. All the stressed samples were concentrated and impurities in the pure form were isolated on preparative LC using 5mM ammonium acetate in water and acetonitrile mobile phase in gradient elution. Each isolated fractions were concentrated and passed through C-18 column on preparative LC using water-ACN to remove ammonium acetate. Each pure impurity fraction was concentrated on rotavapor, dried under high vacuum and stored at -20ºC.

N-acetylated impurity was not observed during stress study and was synthesized in our chemistry laboratory. Sitagliptin API was stirred in DCM at room temperature along with acetyl chloride and triethyl amine for 24 hr and then extracted with ethylacetate. The impurity was then purified on preparative HPLC using water, acetonitrile as mobile phase on a C-18 column. Each isolated degradation product was tested for HPLC purity and found to be >98%. N-acetyl impurity was characterized by NMR and Mass (**Fig. 3E**)

Preparation of Standard and Sample Solutions:

1. **Standard solutions:** Standard stock solution, 1mg/ml prepared by dissolving appropriate amount of sitagliptin phosphate in 100ml volumetric flask using diluent (water: acetonitrile, 9: 1) and stored at 4ºC for three weeks with no evidence of decomposition confirmed by HPLC. Further solutions for linearity were prepared by serial dilution from stock solution.

2. **Sample solutions:** Seven Januvia® 100mg tablets were weighed and finely crushed in a mortar and pestle. Samples were prepared by transferring appropriate amount of tablet powder (equiv. to 10mg free base) to each 10ml volumetric flask and 5ml of diluent added to each flask and sonicated for 15min. Solutions were then made up to the volume using water. Test concentration, 100µg/ml samples were prepared by further diluting above solutions in 10ml volumetric flask with diluent.

RESULTS AND DISCUSSION:

HPLC method development and optimization: The aim of this HPLC method development was to develop a rugged and robust stability indicating method which can separate all existing degradation products in this forced degradation study.

Using a C-18 column, various aqueous mobile phases such as ammonium acetate (5mM, pH 6.7), potassium dihydrogenphosphate (10mM, pH 3, pH 4.5, pH 7), acetic acid (0.1%), formic acid (0.1%), trifluoroacetic acid (0.1%) and o-phosphoric acid (0.1%) in combination with methanol and acetonitrile were evaluated to obtain good peak shape and better sensitivity.

Best possible peak shape and sensitivity was observed with o-phosphoric acid (0.1%) and acetonitrile. Further, method development was initiated to obtain stability indicating method for sitagliptin in presence of spiked degradation products. Due to very polar nature of DP-I and DP-II, it was difficult to get separation with acidic mobile phase, whereas neutral pH mobile phase was a better choice. Water with methanol or acetonitrile had not given proper separation and peak shape. Ammonium acetate (5mM) with methanol and acetonitrile were tried and found good separation with acetonitrile combination on Agilent eclipse XDB-C18 (4.6X250mm, 5µ) column.

Further, sensitivity was improved using sub-micron particle columns, Ascentis® Express F5 10X3mm 2.7µ (Sigma-Aldrich), Ascentis® Express RP-Amide 10X3mm 2.7µ (Sigma-Aldrich), Zorbax Eclipse XDB-C18 RRHT 4.6X50mm 1.8µ (Agilent Technology) and Poroshell 120 EC-C18 2.7µ 3X150mm (Agilent Technology).

Finally poroshell column was selected due to its sensitivity, resolution and reproducibility over other columns. The final chromatogram with spiked impurities and degradation products for the optimized method is given in **Fig. 2**.

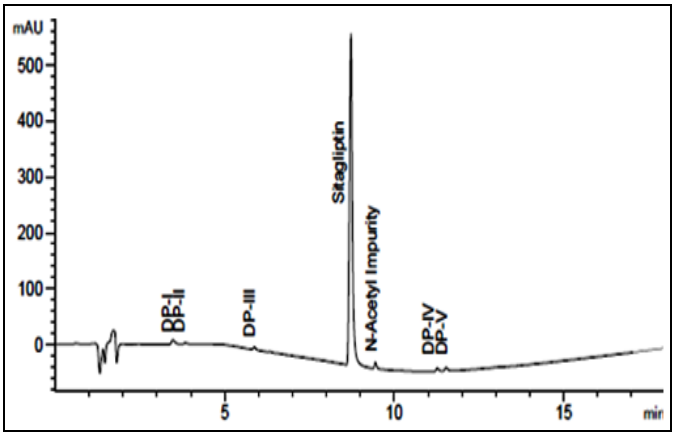


FIG. 2: REPRESENTATIVE CHROMATOGRAM OF SITAGLIPTIN WITH SPIKED IMPURITIES

Degradation study: After conducting sitagliptin API and tablet powder stress studies under various stress conditions, we observed higher degradation of sitagliptin in tablet powder than API samples. **Table 1** summarizes degradation profile of sitagliptin under different stress conditions and at different incubation periods.

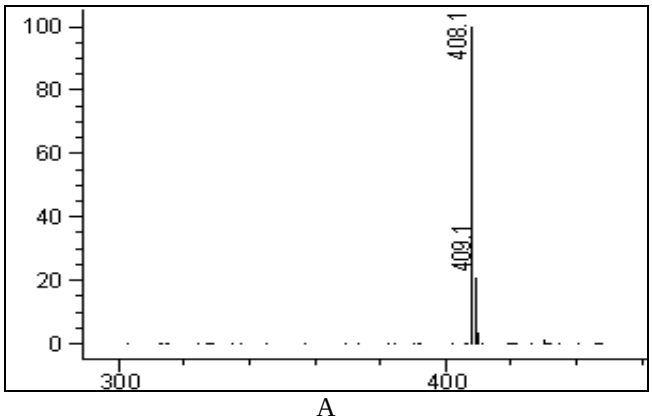
TABLE 1: DEGRADATION PROFILE OF SITAGLIPTIN API AND TABLET POWDER UNDER VARIOUS STRESS CONDITIONS (VALUES SHOWN ARE PERCENTAGE AREAS)

Stress Condition and Sampling Time	API						Tablet Powder					
	Degradation Products					Sitagliptin	Degradation Products					Sitagliptin
	I	II	III	IV	V		I	II	III	IV	V	
0.1N NaOH, 4hr	-	1.5	4.5	-	-	93.9	-	2.3	3.3	-	-	91.6
0.1N NaOH, 24hr	-	6	16.7	-	-	77	-	5.8	17.4	-	-	76.7
0.1N HCl, 55ºC, 2 Days	-	-	1.3	-	-	98.6	-	-	1.3	-	-	97.8
0.1N HCl, 5 Days	-	0.2	3.4	-	-	96.3	-	0.2	3.3	-	-	96.4
0.1N HCl, 2 Weeks	-	1	9.2	-	-	89.6	-	1	8.9	-	-	90
Neutral, 55ºC, 2 Days	-	-	0.3	0.6	-	98.9	-	-	2.2	3.9	-	92.8
Neutral, 55ºC, 5 Days	-	-	0.9	1.7	-	97.2	-	0.2	5.4	9.4	0.4	84.4
Neutral, 55ºC, 2 Weeks	-	-	2.5	4.7	0.1	92.6	-	1	12.3	18.2	2.4	65.8
5%H_2O_2, 55ºC, 1 Day	0.5	-	-	-	-	96.1	1.7	-	-	-	-	79.7
5%H_2O_2, 55ºC, 3 Days	2	-	-	-	-	86.9	6.3	-	-	-	-	57.5
5%H_2O_2, 55ºC, 5 Days	3.6	-	-	-	-	80.3	10	-	-	-	-	46.3
Solid, 80ºC, 10 Days	-	-	-	0.2	-	99.2	-	-	-	0.8	5.4	93.3
UV 254nm, 10 Days	-	-	-	-	-	99.98	-	-	-	-	-	99.98

Structure elucidation of Impurities:

1. **Characterization of DP-I:** The mass of DP-I is 207, which was isolated as an oxidative degradation product (**Fig. 3B**). The ^{19}F NMR spectra gave a distinct peak at δ 64.4, similar to CF_3 group of triazolopyrazine moiety of parent drug. The ^{1}H NMR spectra gave three peaks corresponding to the NH, CH_2, CH_2 and ^{13}C NMR spectra indicated presence of carbonyl carbon.

   This indicated parent molecule has been cleaved at amide bond and triazolopyrazine moiety got oxidized at one of the carbon atom. gCosy NMR spectra showed correlation between two adjacent CH_2 groups confirming the location of carbonyl group.



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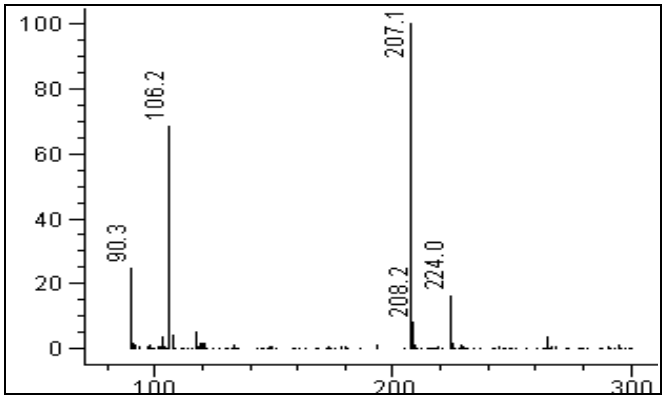

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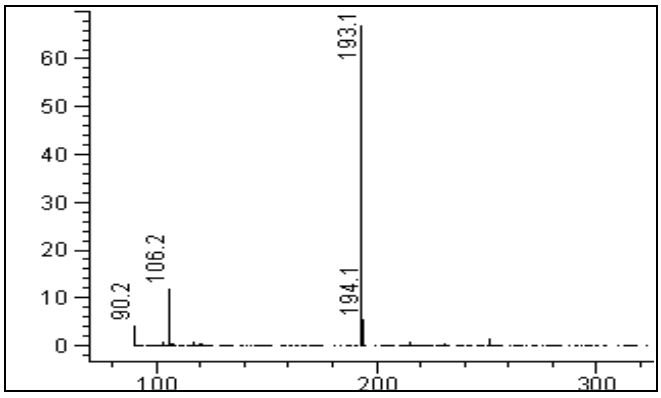
2. **Characterization of DP-II and DP-III:** DP-II and DP-III were isolated as base and acid hydrolysis products in solid form. The molecular weights are 193 and 234 respectively (**Fig. 3C, 3D**), indicated that the products are formed from simple amide bond hydrolysis. Structure of the two products was confirmed from ^{1}H, ^{13}C, ^{19}F and gCosy NMR spectra.



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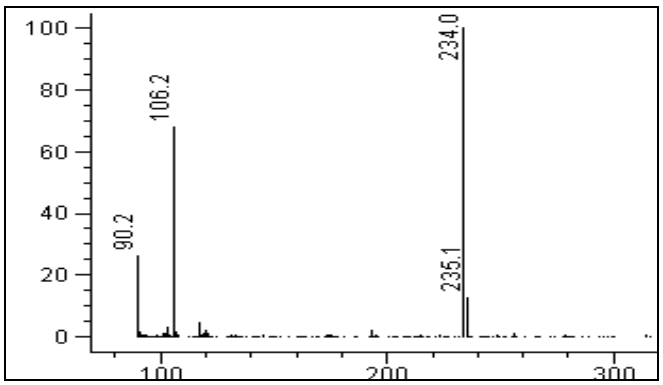

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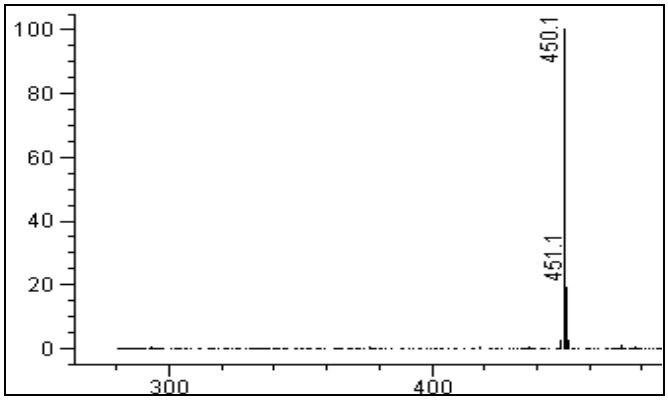

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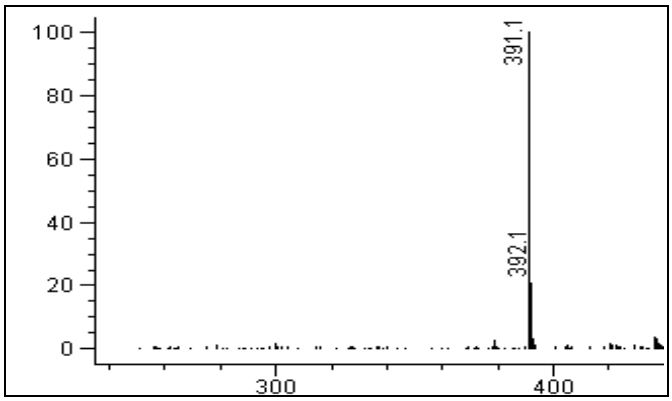

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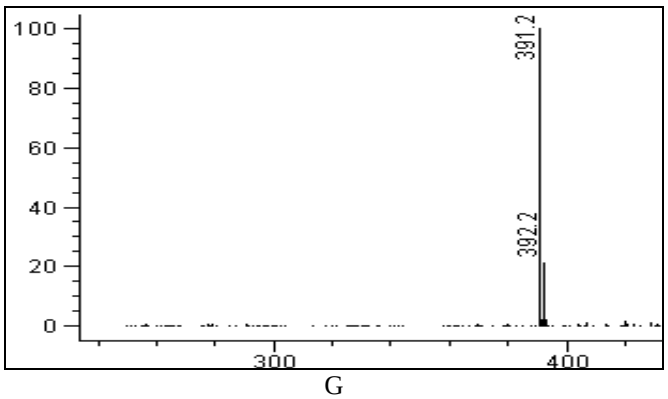

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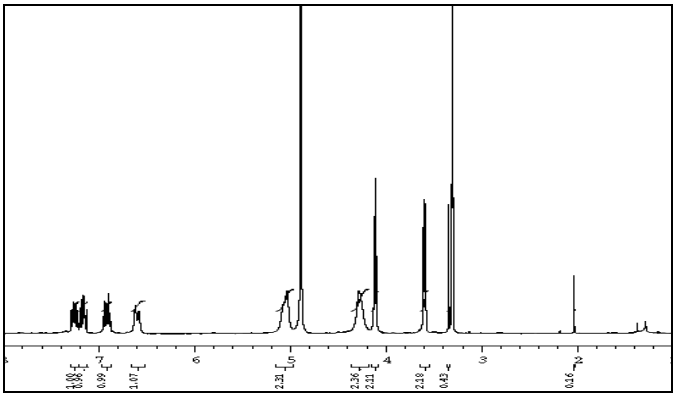
FIG. 3: MASS SPECTRUM'S (M+1) OF (A) SITAGLIPTIN, (B) DP-I, (C) DP-II, (D) DP-III, (E) N-ACETYLATED IMPURITY, (F) DP-IV AND (G) DP-V

Characterization of DP-IV and DP-V: DP-IV and DP-V were isolated as thermal degradation products, from aqueous solution of drug product stored at 130ºC. The mass of both DP-IV and DP-V is 391; indicating products are formed with a loss of mass 17 from the parent drug molecule (**Fig. 3F, 3G**). The even molecular weight of DP-IV and DP-V (390) suggest presence of even number of nitrogen's and formed by the loss of nitrogen from the parent drug molecule with odd molecular weight (407) (**Fig. 3A**).

The ^{1}H NMR spectra of both degradation products found to be similar with respect to number of peaks and chemical shifts. Appearance of two olefinic protons in the region of δ 7-6, disappearance of one of the two CH_2 protons of side chain and retention of ring A and B protons confirm deamination. Further study was required to assign location of double bond formation in two isomeric degradation products.

The gCosy NMR spectra of these degradation products have shown differential correlation of olefinic protons and side chain -CH_2 protons, which indicated DP-IV and DP-V are differing in position of double bond on this side chain.

The 1D Noesy NMR of DP-IV has shown interaction of side chain -CH_2 proton with the olefinic protons as well as one of the aromatic proton but not with any of the ring B protons. Whereas 1D Noesy of DP-V has shown interaction of side chain -CH_2 proton with the olefinic protons as well as -CH_2 protons of the ring B but not with any of the aromatic protons (ring A) (**Fig. 4**). These experiments substantiate the structures shown in **Fig. 5** for DP-IV and DP-V.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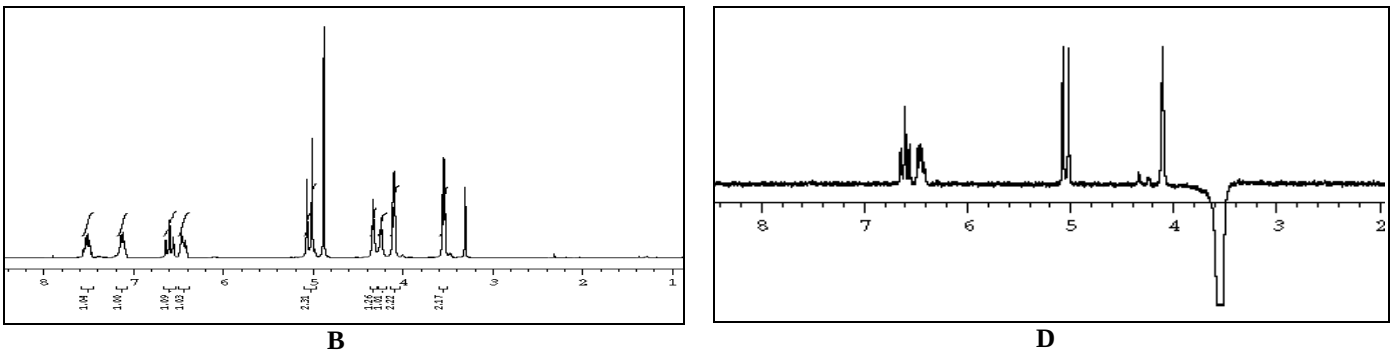

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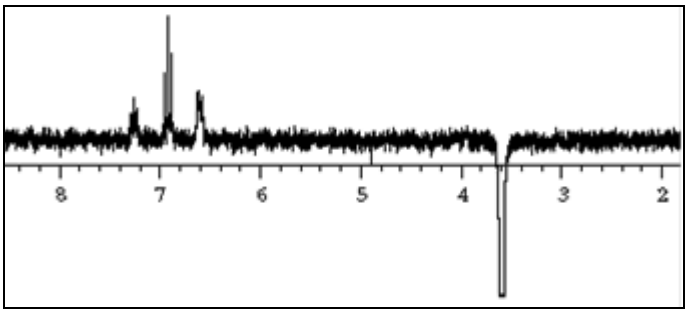

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FIG. 4: NMR SPECTRA RECORDED IN METHANOL-D4 A) PROTON NMR OF DP-IV, B) PROTON NMR OF DP-V, C) 1D NOE OF DP-IV, D) 1D NOE OF DP-V

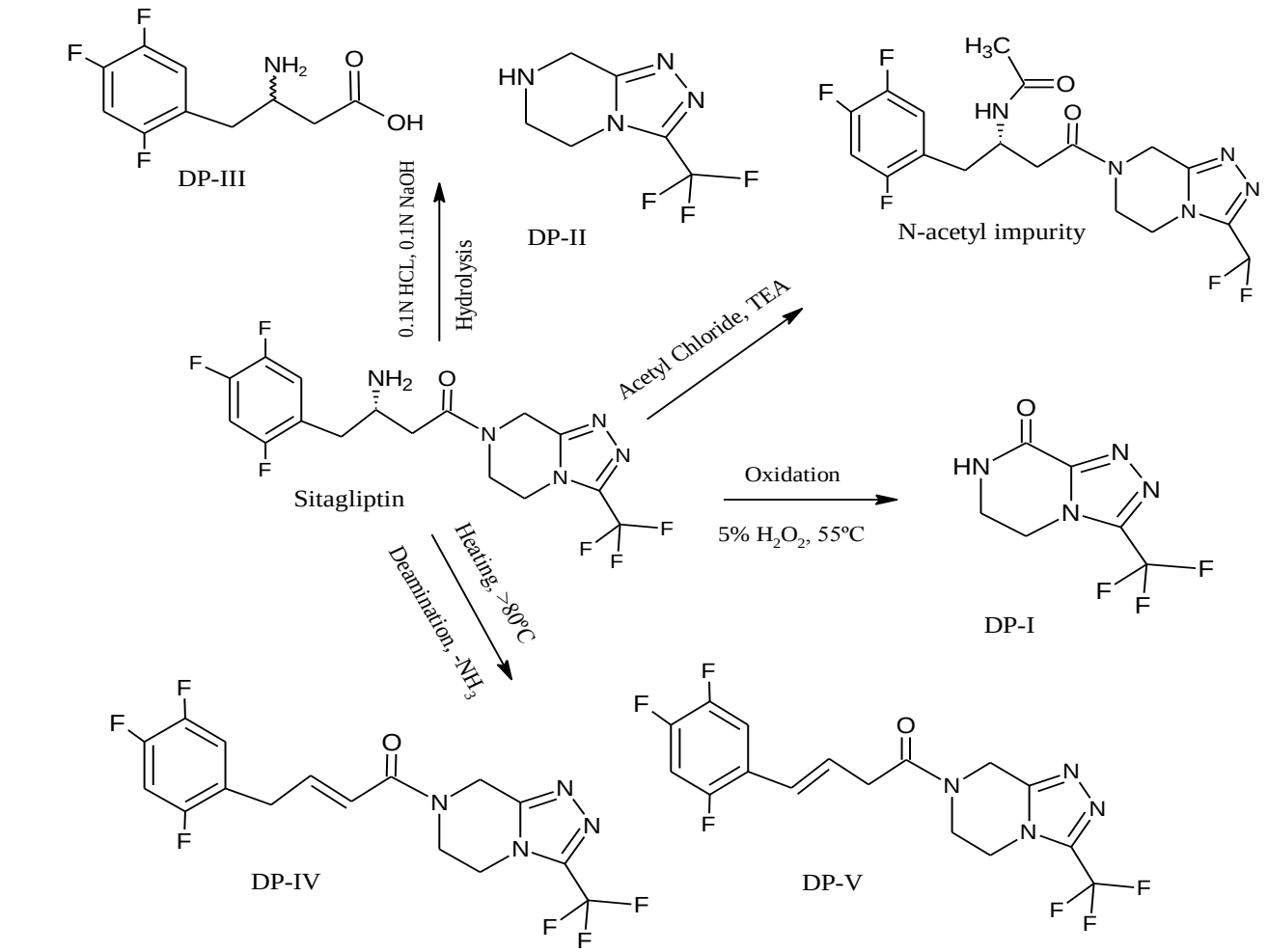

FIG. 5: POSTULATED DEGRADATION PATHWAYS OF SITAGLIPTIN UNDER STRESS CONDITIONS

The comparative proton NMR assignments with respect to Fig. 1 are shown in **Table 2**.

TABLE 2: ASSIGNMENTS OF PROTON NMR CHEMICAL SHIFTS δ (ppm) IN CD_3OD (Refer the structural formula Fig. 1 for proton assignments)

Position of Protons	Proton chemical shifts, δ (ppm)					
	Sitagliptin	DP-I	DP-II[a]	DP-III	DP-IV	DP-V
a	7.3 (1H, m)			7.3 (1H, m)	7.2 (1H, m)	7.5 (1H, m)
b	7.2 (1H, m)			7.2 (1H, m)	7.1 (1H, m)	7.1 (1H, m)
c	3 (2H, m)			2.9 (2H, m)	3.6 (2H, d)	6.6 (1H, t)
d	3.8 (1H, br s)			3.6 (1H, m)	6.9 (1H, m)	6.4 (1H, m)
e	2.9(2H, m)			2.44 (1H, dd)	6.6 (1H, d)	3.5 (1H, t)
				2.29 (1H, dd)		
f	4.9 (2H, m)		4.2 (2H, s)		5 (2H, br s)	5.07 (1H, s)
						5.01 (1H, s)
g	4.2 (2H, m)	4.4 (2H, t)	4 (2H, t)		4.2 (2H, br s)	4.3 (1H, t)
						4.2 (1H, t)
h	4 (2H, m)	3.7 (2H, t)	3.2 (2H, t)		4.1 (2H, t)	4.1 (2H, br s)

[a]$CDCl_3$

Validation of the method: HPLC method validation was performed as per ICH guidelines [18] for the estimation of sitagliptin in presence of degradation products and N-acetyl impurity.

Accuracy: Accuracy of the method was evaluated in triplicate at three concentration levels, i.e. 80%, 100% and 120% of the test concentration (100µg/ml) by spiking known amount of analyte to the tablet powder. The results obtained including the mean recovery and percent RSD are displayed in **Table 3**.

TABLE 3: RECOVERY DATA OF SPIKED SITAGLIPTIN IN TABLET POWDER

Spiked Conc. (µg/ml)	Calculated Spiked Conc. (µg/ml)	R.S.D (%)	Recovery (%)
80	78.63	0.42	98.28
100	99.82	0.18	99.82
120	119.75	0.17	99.79

Precision: The intra and inter-day precision were assessed by using standard solutions prepared to produce six solutions at test concentration of 100µg/ml and percentage RSD values were calculated and summarized in **Table 4**.

Specificity: The result of monitoring standard solution of drug in presence of their spiked degradation products and N-acetyl impurity indicated high degree of specificity of the method. Specificity was confirmed by peak purity studies using PDA detector.

LOQ and LOD: The LOQ was determined as the lowest amount of analyte that was reproducibly quantified. The resultant percent RSD of six replicate injections was found to be 0.4. The LOQ that produced the requisite precision was found to be 1µg/ml. The LOD was 0.5µg/ml, determined based on signal to noise ratio which was three times the background noise.

Linearity: Linearity solutions were prepared from stock solution at six different concentrations from 10µg/ml to 500µg/ml and assessed by injecting the same over three consecutive days. The calibration curve were constructed from the resultant data and found excellent correlation (Correlation Coefficient >0.999) between the peak area and corresponding analyte concentrations. The data are summarized in Table 4.

TABLE 4: DATA FOR SITAGLIPTIN FROM LINEARITY AND PRECISION STUDY

Precision	**Intraday, R.S.D (%)**	**0.13**
	Interday, R.S.D (%)	**1.2**
Linearity	Range of Linearity	10-500µg/ml
	Regression Equation	31.689x+248.47
	Regression Coefficient(r^2)	0.9993
	LOQ (µg/ml)	1µg/ml
	LOD (µg/ml)	0.5µg/ml

Robustness: Robustness of the method was investigated under variety of conditions including change of flow rate, mobile phase composition, column temperature and buffer composition using spiked degradation products along with N-acetyl impurity at 1% of analyte concentration. The analyte was adequately resolved from all the spiked impurities and all the impurities were tolerably resolved from one another. The data representing main peak retention time and resolution with nearest impurity (N-Acetyl) are summarized in **Table 5**.

TABLE 5: ROBUSTNESS DATA AT VARIOUS CONDITIONS

Conditions	Sitagliptin Rt, min	Resolution between drug and nearest peak (impurity)
Final optimized condition	8.7	5.9
Column Temp 45ºC	8.6	5.8
Column Temp 35ºC	8.7	5.9
Acetonitrile 10% high	8.3	5.5
Acetonitrile 10% less	9.1	6.6
Flow 0.55ml/min	8.2	6.3
Flow 0.45ml/min	9.1	5.7
Ammonium acetate 6mM	8.6	6.4
Ammonium acetate 4mM	8.7	5.8

CONCLUSION: Degradation products of sitagliptin were successfully isolated, purified and characterized by mass, NMR and IR spectroscopy. The structures of three novel degradation products were elucidated, which were not reported earlier. Further, a precise, accurate and sensitive mass compatible stability indicating HPLC assay method was developed and validated according to ICH guideline. This method is novel and superior as it involves the separation of three additional novel impurities. The proposed method can be used for the analysis of stability samples of sitagliptin in bulk and tablet formulation.

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