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REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH MASS DETECTION AND NMR CHARACTERIZATION OF SITAGLIPTIN DEGRADATION RELATED IMPURITIES

F. I. Farooqui* and R. B. Kakde

Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur-440033, Maharashtra, India

Keywords:

Sitagliptin, Degradation related impurities, Gradient elution

Correspondence to Author:
F. I. Farooqui,

Research Scholar,
Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur-440033, Maharashtra, India

Email: faizan55_farooqui@yahoo.co.in

ABSTRACT: The current work on impurity profiling of Sitagliptin in its bulk drugs, included development of a stability- indicating reverse-phased liquid chromatographic method and its validation for the estimation of degradation related impurities (DRIs). Sitagliptin was subjected to acid and alkaline hydrolysis, H_2O_2 oxidation, thermal degradation and photolysis. Acid hydrolysis and H_2O_2 oxidation yielded significant degradants, which were isolated on a semi-preparative high performance liquid chromatography (HPLC) and characterized with the help of high-resolution mass spectrometry, ^{1}H-NMR spectroscopy. Possible structures of the DRIs were reveled with the help of mass measurement and NMR spectroscopy. The chromatographic separations were accomplished on Waters Cosmosil C_{18} column (250 mm x 4.6 mm; 5 µm) using 5mM ammonium acetate and methanol as a mobile phase with gradient elution at 1.0 ml/min flow rate, and eluents were detected using photo diode array detector at 268 nm wavelength. The method was validated with respect to accuracy, precision, linearity, robustness, and limits of detection and quantification as per International Conference on Harmonization (ICH) guidelines.

INTRODUCTION: Sitagliptin (**Fig. 1**) is a competitive inhibitor dipeptidyl peptidase-4 (DPP-4)used as an oral hypoglycemic agent for the management of type-II diabetes mellitus. The DPP-4 inhibitors increase the levels of glucagon like peptide-1 (GLP-1), which in turn enhances the insulin production.[1]

A literature study about the analytical studies performed on Sitagliptin, including the spectrometry and high performance liquid chromatography (HPLC) reveals a few selected methods for the determination of Sitagliptin either single or in combination with some other drugs like, metformin, simvastatin, etc. [2, 3, 4] Some methods involve hyphenated techniques such as, LC-MS, LC-MS/MS.[5, 6]

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However, none of these methods have been able to address the impurity profiling of Sitagliptin, i.e. isolation and characterization of the impurities present. The current study is focused on the isolation and characterization of the degradation-related impurities (DRIs) developed as a result of the degradation studies.

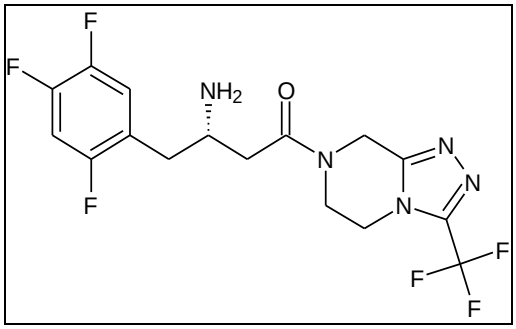


FIG.1: SITAGLIPTIN

MATERIALS AND METHODS:

Chemicals:

Sitagliptin was obtained from Sun Pharma, Baroda, India. HPLC grade Methanol was purchased from Merck chemicals (Darmstadt, Germany). AR-grade

ammonium acetate, hydrochloric acid, sodium hydroxide, hydrogen peroxide, formic acid, were purchased from S.D. Fine Chemicals, India. HPLC grade water utilized from the Milli-Q water purification system, available in the laboratory.

Instrumentation:

HPLC Instrumentation:

Chromatographic work was carried on Prominence UFLC LC-20 system with a photo diode array (PDA) detector (Shimadzu, Japan), using LC-solution software (Shimadzu, Japan) for system control and data acquisition. Chromatographic separation was obtained using Cosmosil C_{18} column (250 mm X 4.6 mm; 5µm; Waters, USA). The mobile phase was composed of 5mM ammonium acetate (Solvent A) and methanol (Solvent B) in a gradient elution, with a flow rate of 1.0 ml/min. The gradient elution was programmed as; time/percentage of Solvent B – 0/20, 3/20, 15/40, 28/10, with an equilibrium time of 2 min. The injection volume was 20µl and the eluents were monitored at 268 nm.

Semi Preparative HPLC instrumentation:

The isolation of the DRIs was carried out using Shimadzu semi-preparative HPLC system, (Shimadzu, Japan). The system consisted of two gradient LC-pumps, a PDA detector (Shimadzu, Japan) with a flow cell of 10 mm and a fraction collector. The system was controlled by Lab-solution software (Shimadzu, Japan) for the data acquisition and time programming. For the isolation of the DRIs, semi-preparative column Enable HPLC C_{18} (250 X 10 mm; 10µm) was used, with a simple gradient mobile phase of 5mM ammonium acetate (Solvent A) and methanol (Solvent B). The separation was performed with gradient elution program: time/percentage of solvent B, 0/10, 5/40, 10/50, 17/10, with an equilibrium time of 3 min. The injection volume was 2ml, with a flow rate of 8 ml/min, and detection at wavelength 268 nm.

Preparation of Sample Solution:

Stock solutions of Sitagliptin and its DRIs were prepared by dissolving known amounts of components in methanol. The specification concentration of Sitagliptin was 1000 µg/ml for DRIs and 100µg/ml for the assay determination. For the sample loading in semi-preparative HPLC the concentration taken was, 30,000µg/ml.

Forced Degradation Studies:

Forced degradation studies were performed on Sitagliptin, for which 1.0 mg/ml concentration of sample was used. Samples were withdrawn at particular intervals, and injected to monitor the degradation process.

Acid Degradation studies:

The sample solutions for acid degradation were prepared in 1N hydrochloric acid (HCl) and kept at 60˚C, for 6 hours. Samples were withdrawn at regular intervals for monitoring the degradations.

Alkaline Degradation studies:

The sample solutions for alkaline degradation were prepared in 1N sodium hydroxide (NaOH) and kept at 60˚C, for 6 hours. Samples were withdrawn at regular intervals for monitoring the degradations.

Oxidation Degradation studies:

The sample solutions for oxidative degradation were prepared in 30% hydrogen peroxide (H_2O_2) and kept at room temperature for 10 days. Samples were withdrawn at regular intervals for monitoring the degradations.

Thermal Degradation studies:

The samples for thermal degradation were kept in oven at 80˚C for 10 days. Samples were withdrawn at regular intervals for monitoring the degradations.

Photo Stability studies:

The samples were exposed to light using a petri-dish for 10 days. Samples were taken at regular intervals to monitor degradations.

Results and Discussion:

Method Development and Optimization:

Waters Cosmosil C_{18} column (250 X 4.6 mm; 5µm) was finalized as the column for this study, after trial and error runs with other columns like, C_8, phenyl and cyano. For the optimization of the column different mobile phase combinations involving methanol, acetonitrile, buffer solution (Ammonium acetate) were tried, in isocratic and gradient modes.

Finally, good separation and peak shape where observed with the use of 5mM ammonium acetate and methanol, in a gradient mode of elution. The DRIs were optimized on the same developed mobile phase, and a chromatogram of Sitagliptin along with its DRIs was observed as given in **Fig.2**.

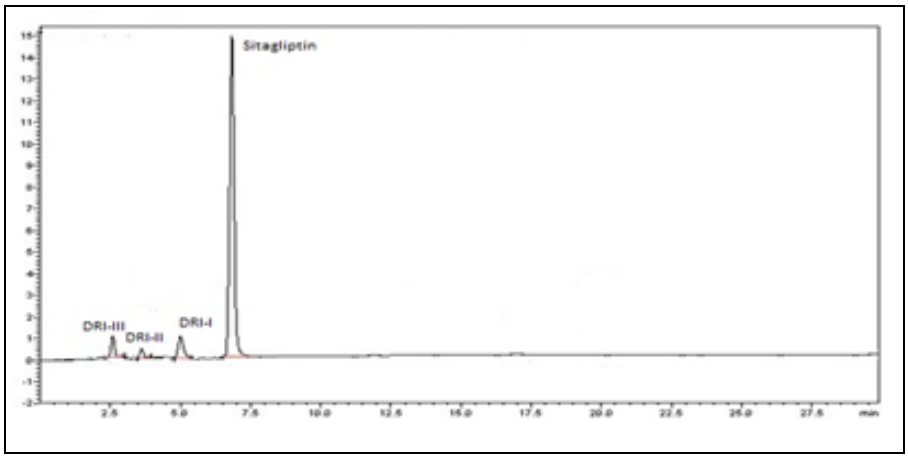


FIG. 2: CHROMATOGRAM OF SITAGLIPTIN SPIKED WITH 1% DRIs FORCED DEGRADATION STUDIES:

The forced degradation studies were performed on Sitagliptin under different stress conditions, yielding three important degradation related impurities (DRIs) of Sitagliptin. The results of the degradation studies have been tabulated below, in **Table 1.**

TABLE 1: FORCED DEGRADATION STUDIES RESULTS FOR SITAGLIPTIN AND ITS DRIs

Stress Condition	Time	Sitagliptin Assay (%w/w)	DRI (%w/w)	Remark
Acid Hydrolysis, HCl 1N, 60˚C	6 hours	77.12	15.27 (DRI-I) 5.06 (DRI-II)	DRI-I and DRI-II
Base Hydrolysis, NaOH 1N, 60˚C	6 hours	96.63	-	No DRI
Oxidation, 30% H_2O_2	10 days	82.91	7.94 (DRI-III)	DRI-III
Thermal, 80˚C	10 days	99.01	-	No DRI
Photo-Stability	10 days	99.97	-	No DRI

Structural Elucidation:

DRI-I and DRI-II:

DRI-I (**Fig. 3**) and DRI-II (**Fig. 4**) were isolated as acid hydrolysis products. The elemental composition of DRI-I eluting at retention time (Rt) 2.61 min was $C_{10}H_{10}F_3NO_2[M+H]^+$, having a molecular mass of 233.1, which suggested hydrolysis of an amide bond. Similarly, for DRI-II with Rt 5.06 min the elemental composition was $C_6H_{11}F_3N_4$ $[M+H]^+$, with a molecular mass of 196.3 again suggests amide bond hydrolysis. The structures of these two DRIs were confirmed with the help of ^1H NMR and ^{13}C NMR spectra. From the spectral data the DRI-I and DRI-II were confirmed as, 3(S)-3-amino-4-(2, 4, 5-trifluorophenyl) butanoic acid and 3-(trifluoromethyl) - 5, 6, 7, 8- tetrahydro[1, 2, 4] triazolo [4,3-a] pyrazine, respectively.

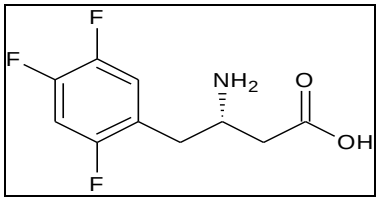


FIG.3. DRI-I

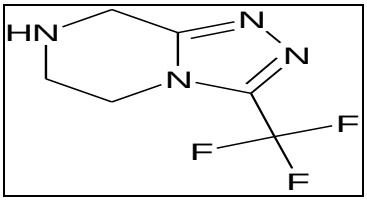


FIG. 4. DRI-II

DRI-III:

DRI-III (**Fig. 5)** was isolated as oxidative degradation product of Sitagliptin. The elemental composition of DRI-III eluting at Rt 3.53 min was $C_6H_5F_3N_4O$ $[M+H]^+$, having molecular mass of 206.3. The ^1H NMR for DRI-III shows the

presence of CH2, CH2, and NH groups. While ^{13}C NMR indicates a carbonyl carbon. From the ^{1}H and ^{13}C NMR it was deduced that the oxidation of the triazolopyrazine moiety occurred at one of the carbon atoms in the parent molecule. Thus DRI-III was finally confirmed as, 3- (trifluoromethyl)-6, 7-dihydro [1, 2, 4] triazolo [4, 3-a] pyrazine-8(5H)-one.

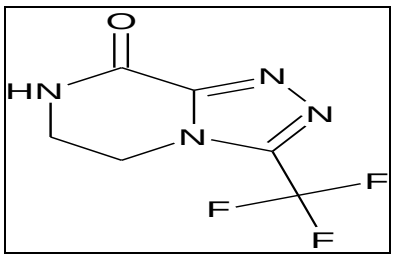
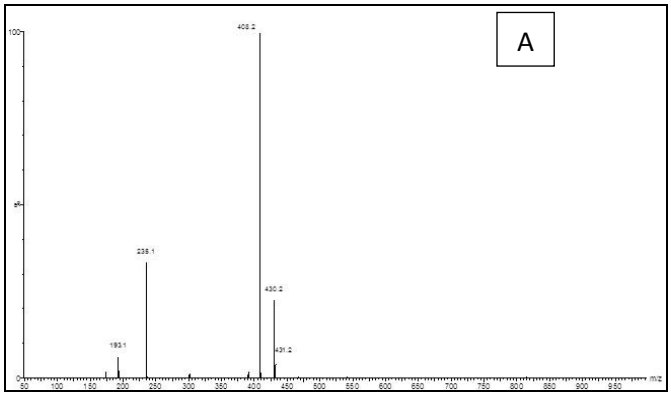
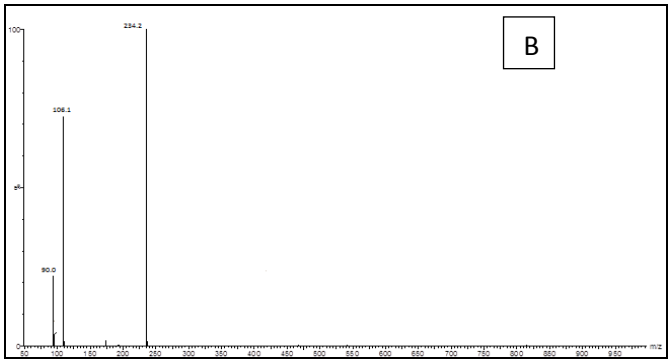
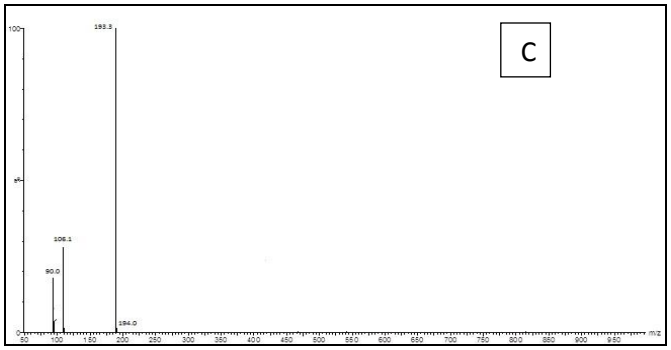


FIG.5: DRI-III







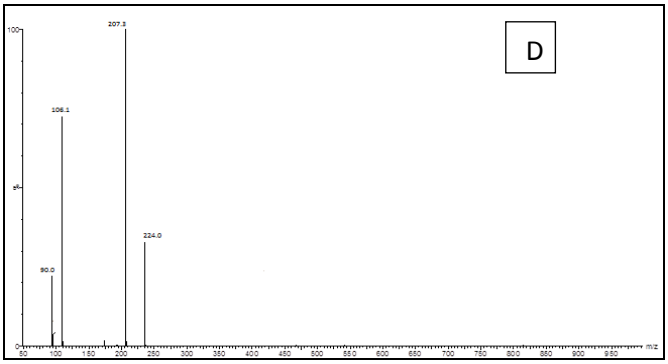


FIG. 6: MASS SPECTRA OF (A) SITAGLIPTIN, (B) DRI-I, (C) DRI-II, (D) DRI-III.

TABLE 2: ^{1}H NMR ASSIGNMENTS FOR SITAGLIPTIN AND ITS DRIs

Position	Sitagliptin	DRI-I	DRI-II	DRI-III
1	2.805 (m)	2.992 (d)	3.255 (t)	3.764 (t)
2	3.073 (m)	2.413 (d)	4.057 (t)	4.402 (t)
3	3.852 (s)	2.941 (m)	4.281 (s)	
4	4.039 (m)	3.605 (m)		
5	4.250 (m)	7.302 (m)		
6	4.906 (m)	7.998 (m)		
7	7.331 (m)			
8	7.927 (m)			

Method Validation:

To make sure that the developed method is reproducible and reliable, it was validated as per the ICH guidelines.[7, 8, 9]

Accuracy:

For accuracy studies, recovery experiments were performed in triplicate at three concentrations, i.e., 50%, 100% and 150% of impurities spiked to Sitagliptin (1000µg/ml). For assay method, accuracy was studied at three concentration levels of 50µg, 100µg and 150 µg, respectively. The results for the accuracy are given in the **Table 3**.

TABLE 3: ACCURACY RESULTS

Parameters	Sitagliptin	DRI-I	DRI-II	DRI-III
Accuracy (50%)				
Amount added (µg/ml)	25	0.75	0.75	0.75
Amount recovered (µg/ml)	24.68	0.759	0.737	0.771
Percentage Recovery	98.72	101.10	98.26	102.80
% RSD	0.064	1.171	0.068	0.486
Accuracy (100%)				
Amount added (µg/ml)	50	1.5	1.5	1.5
Amount recovered (µg/ml)	50.429	1.535	1.482	1.497
Percentage Recovery	100.84	102.00	98.66	99.33

% RSD	0.372	1.193	0.076	1.597
Accuracy (150%)				
Amount added (µg/ml)	75	2.25	2.25	2.25
Amount recovered (µg/ml)	75.18	2.23	2.27	2.22
Percentage Recovery	100.24	99.11	100.83	98.69
% RSD	0.086	0.632	0.325	0.477

Precision:

The precision studies were carried out by injecting six replicates of standard solution for Sitagliptin (100µg/ml), and for the DRIs by spiking 0.15% of each DRI with Sitagliptin (1000µg/ml). The % RSD of each DRI were calculated and tabulated in **Table 4**.

TABLE 4: PRECISION RESULTS

Parameters	Sitagliptin	DRI-I	DRI-II	DRI-III
System Precision (%RSD)	0.984	1.604	1.183	1.678
Method Precision (%RSD)	0.315	0.978	0.635	1.327

LOD and LOQ:

The LOD and LOQ of Sitagliptin and the three DRIs were estimated at signal to noise ratio (S/N) of 3:1 and 10:1, respectively, by injecting a series triplicate injections with known concentrations. The results of which are given in **Table 5**.

TABLE 5: LOD AND LOQ RESULTS

Parameters	Sitagliptin	DRI-I	DRI-II	DRI-III
LOD (µg/ml)	0.03	0.08	0.05	0.05
LOQ (µg/ml)	0.10	0.25	0.15	0.15

Linearity: The linearity test solutions of Sitagliptin and its DRIs were prepared at ten different concentration levels ranging from LOQ to 250% of the specification level (Sitagliptin 1000µg/ml). The peak area vs concentration was studied and co-efficient of regression (r^2) values were ≥ 0.9940, there by showing good linearity of the method. The data for linearity is summarized in **Table 6**.

TABLE 6: LINEARITY RESULTS

Parameters	Sitagliptin	DRI-I	DRI-II	DRI-III
Range (µg/ml)	10 - 100	0.2 - 2.5	0.2 - 2.5	0.1 - 2.0
Co-efficient of Regression (r^2)	0.9998	0.9993	0.9986	0.9940

Robustness:

Robustness of the developed method was evaluated by changing flow rate by 10% (1.0 ml ± 0.1 ml), mobile phase composition and column temperature. By evaluating all these chromatographic conditions, the resolution between the analytes was found to be good, while the tailing factor of each analyte was below 1.2; indicating the robustness of the method.

CONCLUSION: Three major Degradation related impurities of Sitagliptin were successfully isolated by semi-preparative HPLC and characterized with the help of Mass and NMR spectroscopy. Furthermore, a simple gradient RP-HPLC method was developed and validated for the determination of Sitagliptin and its degradation related impurities, as per the ICH guidelines.

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CONFLICT OF INTEREST: The authors have declared no conflict of interest.

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