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DEVELOPMENT AND VALIDATION OF SIMPLE EFFECTIVE HPLC METHOD FOR THE QUANTITATIVE DETERMINATION OF RELATED SUBSTANCE PRESENT IN SITAGLIPTIN PHOSPHATE DRUG SUBSTANCE

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Key words:

Sitagliptin related substance, HPLC method development, Method validation, Degradation impurities

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ABSTRACT: An efficient and sensitive RPHPLC method was developed and validated for the estimation of Sitagliptin Phosphate process impurities in drug substances which were identified and characterized by LCMS, FTIR, 1H NMR, C NMR techniques. The method was carried out on a Symmetry shield RP 18 column at 25°C using a 1 % Perchloric acid and acetonitrile in gradient mode of pump. The flow rate is 1.2 ml/min and detection was done at 210nm.The developed RP HPLC method was validated by testing specificity, precision, Forced degradation, detection limit, quantification limit, linearity ,accuracy, robustness and range. The linearity of the method was confirmed over the range of 0.113 to 3.384µg/ml for Sitagliptin Phosphate impurities with correlation coeffients greater than r=0.999.The accuracy of the method was found to be 98.5 to 101.1% and %RSD as found to be less than 2% indicating high degree of accuracy and precision for the proposed method. The effective recovery and lower RSD proves the highness of the proposed RP HPLC method for the routine determination of Sitagliptin Phosphate impurities in drug substances.

INTRODUCTION: Sitagliptin Phosphate is described chemically as 7-[(3R)-3-amino-1-OXO-4-(2,4,5-trifluorophenyl)butyl]-5,6,7,8 - tetrahydro-3-(trifluoromethyl)-1,2,4-triazolo [4,3-a] pyrazine, monophosphate **(Fig.1**). Sitagliptin is used for the treatment of type 2 diabetis. It is effective in lowering of HbA1c, fasting as well as postprandial glucose in monotherapy and in combination with other oral anti diabetic agents. It stimulates insulin secretion when hyperglycemia is present and inhibits glucagon secretion. Sitagliptin is a highly selective DPP-4 inhibitor that has approved for type2 diabetes therapy.[1-6]

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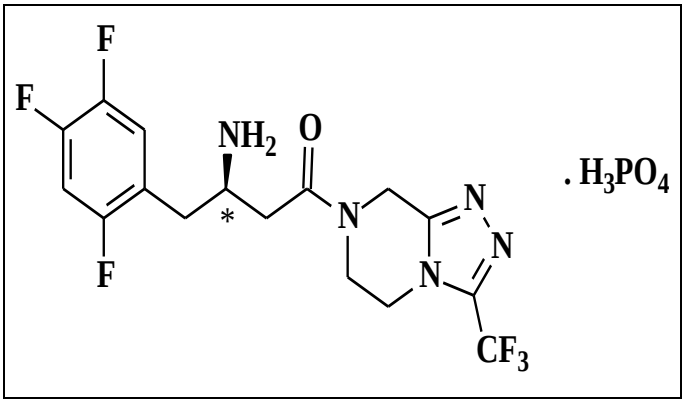
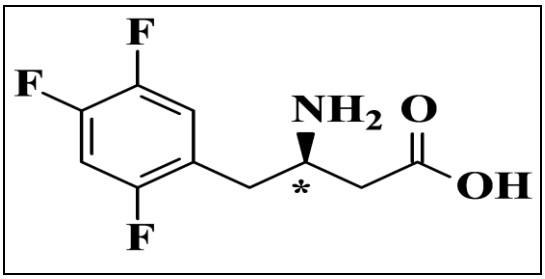


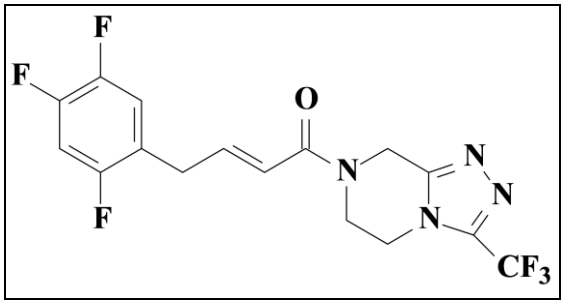
FIG.1: CHEMICAL STRUCTURE OF SITAGLIPTIN

The present research work was to developed a suitable stability indicating gradient HPLC method for the determination of Sitagliptin Phosphate related substances/impurities present in drug substances **(Fig.2**). Both the United states Pharmacopoeia (USP) and the European Pharmacopoeia (EP) have not published monographs for this drug substances.

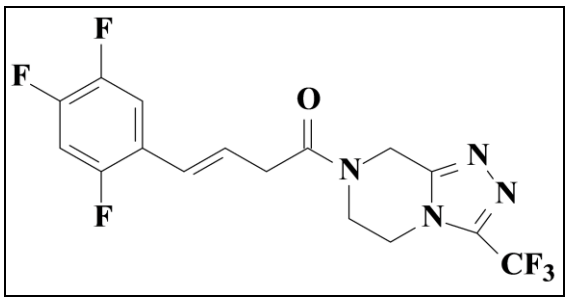
However many analytical methods have been reported in literature on the determination Sitagliptin with combination of other anti diabetic drug like Metformin hydrochloride [7]. Mostly the analytical methods are reported based on UV [8-10], Spectroflourimetry [11] and reverse phase HPLC techniques [12-13]. There are some methods which are used to determine Sitagliptin in human plasma and Urine by LC-MS/MS and GC-MS 14-17.But no literature has been reported on determination of Sitagliptin phosphate impurities present in API or in drug product.



SITAGLIPTIN IMPURITY-1



SITAGLIPTIN IMPURITY-2



SITAGLIPTIN IMPURITY-3

FIG. 2: CHEMICAL STRUCTURES OF SITAGLIPTIN IMPURITES/RELATED SUBSTANCES

Hence the method described in this paper was developed to estimate impurities of Sitaglipin very accurately at the level 0.113µg/mL and also the method is linear to determine sitagliptin phosphate impurities in the range of 0.113 to 3.384 µg/mL, specific and has the ability of good separation of each impurity from Sitagliptin (**Fig.2**).

The developed method was validated for it's routine use of estimation of impurities in quality control laboratories with respect to specificity, limit of detection and quantification, linearity, precision, accuracy, robustness and forced degradation/ stress studies. The stress studies of the drug substance can guide to indicate the likely degradation impurities of the products which can in turn help to establish degradation path ways and the intrinsic stabilities of the molecule. This stability indicating validation of HPLC method was followed in accordance with ICH guide lines [ICH text on validation of analytical procedures QII A (R1) international conference on hormonalisation IFPMA, Geneva]. [18-19]

Experimental:

Chemicals, Reagents and sample:

The investigated Sitagliptin Phosphate and its impurities were taken from APL Research centre II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). Perchloric acid, acetonitrile, Methanol were procured from Merck (India) and Fluka limited and pure Milli-Q water was prepared with help of Millipore purification system.

High performance liquid chromatography (HPLC):

Chromatographic separations were performed on HPLC system with Waters alliance2695 separation module equipped with 2998 photodiode array detector with Empower pro data handling system(Waters Corp., Milford,MA01757,USA) the analysis was carried out on Symmetry shield RP 18, 150 mm × 4.6 mm, 3.5m particle size column. Mobile phase A was 1 % perchloric and mobile phase B was acetonitrile. Diluent was prepared by mixing of water and acetonitrile in the ratio of 80:20(v/v). Injection volume was 20µl, flow rate 1.2 ml/min and column oven temperature 25°C.UV detection was carried out 210nm and data

acquisition time was 65 min. The gradient programe was as follow:

Time(min)/A(v/v): B (v/v); T0.01/95:05,T30/85:15, T50/60:40, T65/35:65, T67/95:05, T75/95:05.

Preparation of standard solutions:
A stock solution of Sitagliptin (450 µg/mL) was prepared by dissolving appropriate amount of substance in the diluent. Working solution of 2.25µg/mL was prepared from this stock solution for the related substance determination.

Sample solution:
Prepared a concentration of 1500 µg/mL of sample solution with diluents.

Method validation:
Specificity:
Specificity is the capability of the method to measure the analyte response of its potential impurities. The specificity of developed HPLC method for Sitagliptin Phosphate was carried out in the presence of its impurities i.e imp-1, imp-2 and imp-3 **Table 2** and also verified the blank interference **Fig [2b]** for the accurate measure of impurities. As a part of specificity stress studies were carried out for Sitagliptin Phosphate drug substance under stress conditions like oxidation,10% H2O2, acid, base, Photolytic(White fluorescent light, 10K Lux and UV light, 200watt-hr/m2), thermal (105°C) and humidity (at 90% RH/25°C) according to ICH option 2 of Q1B. These stress samples were analysed by HPLC using this proposed method at test concentration (1500 µg/mL) to exhibit the ability of the method to separate all three sitagliptin impurities along with its degradation impurities at a quantification level. In these stress conditions the sample's peak purity test was carried out for the sitagliptin Phosphate peak by using PDA.

Linearity, Limits of detections and quantification: Linearity was checked for the related substances method over the concentrations of LOQ, 20%, 25%, 50%, 75%, 100%, 125% and 150% with respect to the 0.15% of the specification level. Linear calibration plot for the related substances method was obtained over the range tested like LOQ to 150 % for the Imp-1, Imp-2 and Imp-3.

The limit of detection (LOD) and limit of quantification (LOQ) of imp-1, imp-2 and imp-3 were determined using the values of slope and standard deviation of responses of individual impurities that have been obtained from the above linearity study with known concentrations.

Accuracy:
The accuracy study of the impurities were carried out in triplicate at LOQ, 50%,100%, and 150% of sitagliptin Phosphate analyte (1500 µg/mL) with respect to the 0.15% level of specification. The sample which was used for validation work, do not show the presence of imp-1, imp-2 and imp-3. Standard addition and recovery experiments were conducted to determine the accuracy of the related substance method for the quantification of all three impurities in the drug substances sample. The study was performed out by spiking each impurity at LOQ, 0.075, 0.15, and 0.225% in the sample solution (1500 µg/mL). The percentages of recoveries for imp-1, imp-2 and imp-3 were calculated from the amount added and amount found values.

Precision:
The precision of the related substances method was performed by two analysts individually by injecting six preparations of sitagliptin precision sample of test concentration of 1500 µg/mL with spiking of imp-1, imp-2 and imp-3 at 0.15% level of analyte concentration. The % of RSD of area for each imp-1, imp-2 and imp-3 were calculated.

Robustness:
To establish the robustness of the method, experimental conditions were deliberately changed, and the plate count, tailing for sitaglitin and the resolution between impurity 2 and impurity 3 was evaluated. The flow rate was 1.2 mL/min. To study the effect of flow rate on the plate count, tailing and resolution, flow rate was changed by ±0.2units from 1.08 to 1.32 mL/min. The effect of the column temperature on plate count, tailing and resolution was studied 20°C and 30°C instead of 25°C. The effect of wavelength on plate count, tailing and resolution was studied by changing the

wavelength -3 nm and +3 nm. The effect of the percent organic strength on plate count , tailing and resolution were studied by varying % of organic in mobile phase gradient composition by -2% and +2% absolute and also changing % of water in mobile phase B by-2% and +2% absolute by keeping the remaining method conditions constant as mentioned in the method. Results are tabulated.

Solution stability:

The solution stability of sitagliptin Phosphate and its impurities in the related substances method was carried out by leaving spiked test solutions in tightly capped volumetric flasks at room temperature for 24hr. Contents of imp-1, imp-2 and imp-3 were determined for every 1hr interval up to 24hr. Contents of imp-1, imp-2 and imp-3 were evaluated in the test solutions.

RESULTS AND DISCUSSION:

Method Optimization by Different Experiments:

The method was optimized by systematic approach by conducting different kind of experiments as a part of Quality-by-design (QbD), as defined by the ICH guideline Q8 9 (R2) [20]. The important aspect of HPLC method is to separate sitagliptin Phosphate peak from imp-1, imp-2 and imp-3.

Impurities were co eluted using different stationary phases such as C8 and C18 and also different mobile phase which containing buffers such as phosphate and perchlorate with different pH(2-6) and using organic modifiers like acetonitrile and methanol in the mobile phase.

Trail-1: Used Di Ammonium hydrogen phosphate buffer (0.01M) with pH 6.0 and acetonitrile as organic modifier and using Zorbax SB C8, 250mm x 4.6mm, 5µ

With flow rate 1.0 ml by following gradient program time (min)/A(v/v): B(v/v); T0.01/80:20, T30/50:50, T40/30:70, T60/30:70, T62/80:20, T70/80:20.

In these conditions 2, and 3 impurities are co eluted with sitagliptin.

Trail-2: Used 0.1% perchloric acid and acetonitrile as organic modifier and using Symmetry shield RP 18, 250 mm × 4.6 mm, 5µ With flow rate 1.0 ml by following gradient program time(min)/A(v/v): B(v/v); T0.01/80:20, T25/70:30, T35/50:50, T45/30:70, T60/30:70, T62/80:20.

But these conditions also not given good separation among the impurities of sitagliptin.

Trail-3: Used 0.1% perchloric acid and acetonitrile and methanol(50:50) as organic modifier and using Symmetry shield RP 18, 150 mm × 4.6 mm, 3.5µ with flow rate 1.0 ml by following gradient program time(min)/A(v/v):B(v/v); T0.01/95:5, T25/90:10, T50/50:50, T60/30:70, T65/30:70, T67/95:5.

These conditions shown all impurities are separated but resolution was not satisfactory.

Hence after fine tuning of trail no 3 we have arrived the final proposed method with well separation of all impurities with good resolution.

Finally the HPLC method was achieved with good separation of sitagliptin Phosphate from imp-1, imp-2 and imp-3 using Symmetry shield RP 18, 150 mm × 4.6 mm, 3.5m column. The mobile phase consists of 1% perchloric acid in water and mobile phase B was acetonitrile with gradient program time(min)/A(v/v):B(v/v); T0.01/95:05, T30/85:15, T50/60:40, T65/35:65, T67/95:05, T75/95:05.

After observing number of experiments by using this method kept the system suitability criteria as the method has to meet column efficiency not less than 10000 plate count and tailing should be not more than 2.0 for sitagliptin peak and also it has to meet the resolution between sitagliptin impurity -2 and sitagliptin impurity-3 is not less than 2.0 in the system suitability injection **(Fig. 3, 4) Table 1.**

The retention time of sitagliptin is about 29 min and the RRT of imp-1, imp-2 and imp-3 are 0.38, 1.61, 1.65 respectively **(Fig . 5) Table 2.**

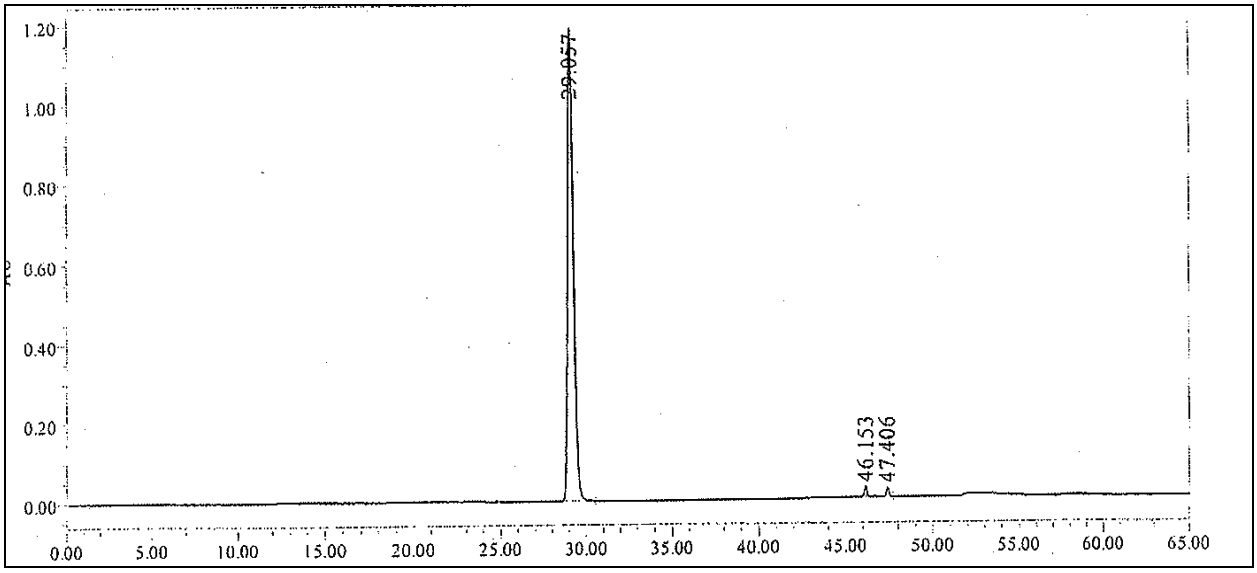


FIG .3: SITAGLIPTIN SYSTEM SUITABILITY CHROMATOGRAM

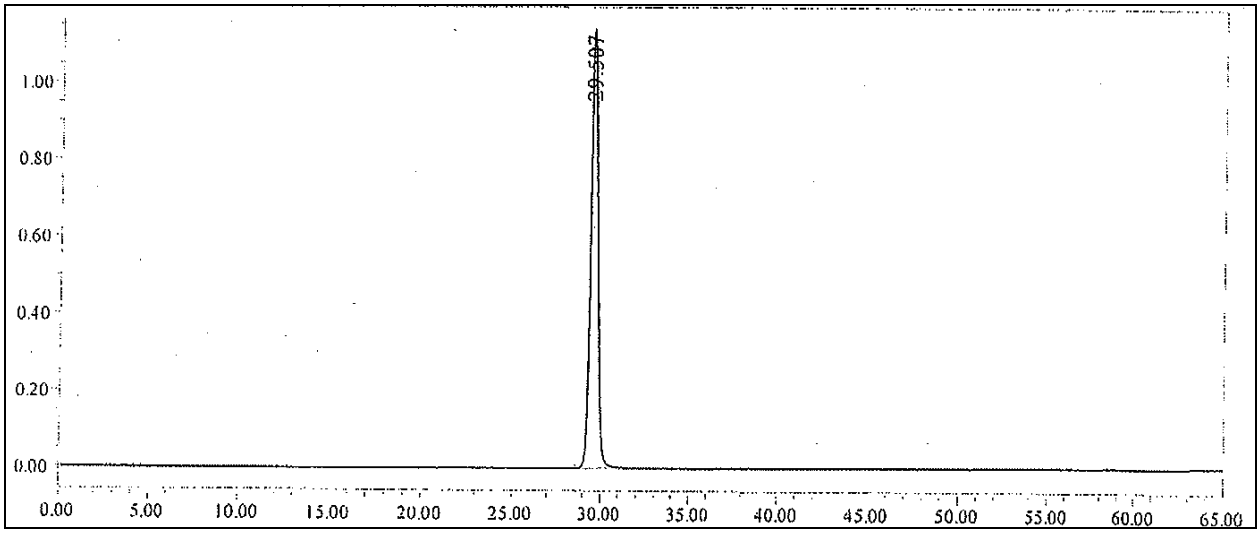


FIG. 4: TYPICAL STANDRAD CHROMATOGRAM OF SITAGLIPTIN

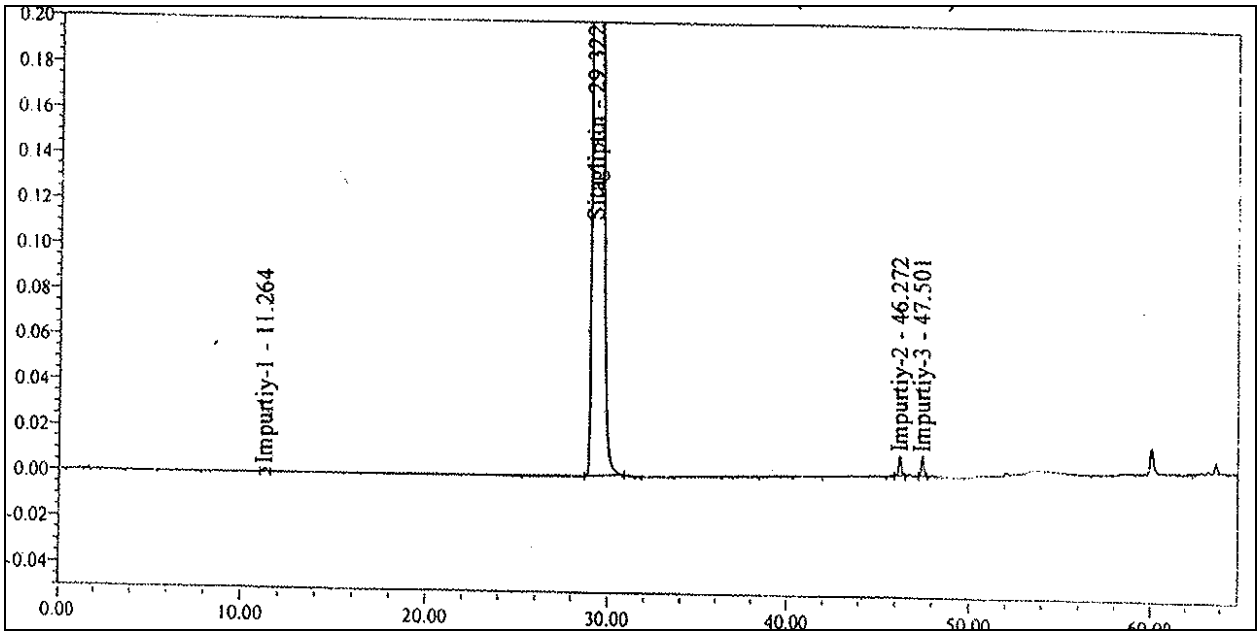


FIG .5: TYPICAL CHROMATOGRAM OF SITAGLIPTIN WITH SPIKED IMPURITIES

TABLE 1: METHOD SUITABILITY PARAMETERS

Compound	RT ratio	USP Resolution	USP plate count	USP Tailing
Sitagliptin	1.00	-	34153	1.5
Sitagliptin Impurity-2	1.61	-	-	-
Sitagliptin Impurity-3	1.65	4.8	-	-

TABLE 2: SPECIFICITY OF SITAGLIPTIN HPLC METHOD

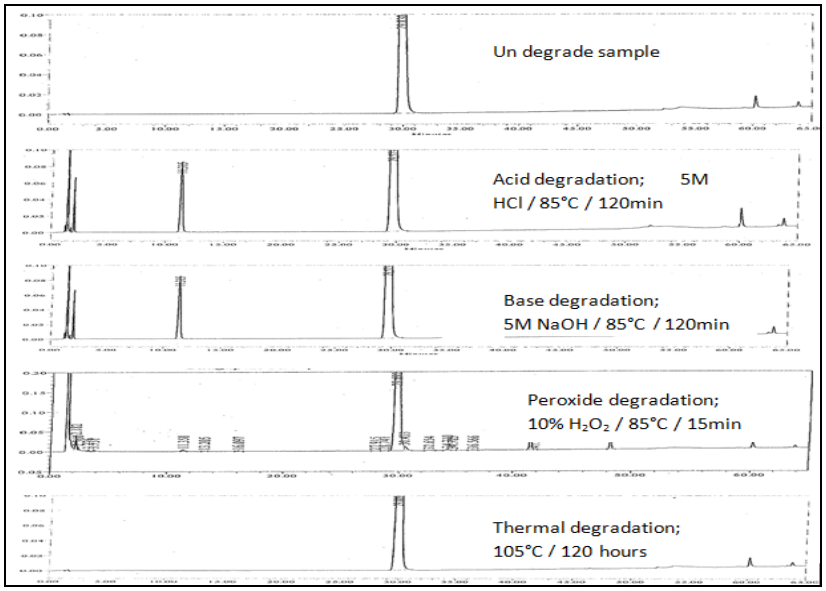
Compound	RT ratio	USP Resolution	USP plate count	USP Tailing
Sitagliptin Impurity-1	0.38	-	18082	1.08
Sitagliptin	1.00	36.05	32456	1.46
Sitagliptin Impurity-2	1.61	36.58	518860	0.96
Sitagliptin Impurity-3	1.65	4.76	589866	1.04

Results of forced degradation:

Sitagliptin Phosphate was susceptible to degradation under stress condition such as acid stress, basic stress and oxidative stress conditions, but considerable degradation of the drug substances was observed under acid, base and oxidative stress conditions **(Fig .6).** Where one major degradation product at 0.31RRT was reported and it's mass is 233 m/z. From the peak purity test results obtained in the stressed drug substances sample, the purity threshold is greater than purity angle; this confirms that the Sitagliptin peak is homogeneous and pure in all the stress samples analyzed. The assay of Sitagliptin Phosphate is unaffected by the presence of imp-1, imp-2 and imp-3 and its degradation products where the mass balance of the undergrad and stressed sample assay value shows the stability-indicating power of the developed HPLC method **Table 3.**

TABLE: 3 SUMMARY OF FORCED DEGRADATION

Stress Condition	Time	% Assay	% Degradation	Peak Purity		% Mass balance	Conclusion
				Purity angle	Purity threshold		
Undegraded	-	99.6	-	0.088	0.446		
Acid hydrolysis (5 M HCl)	85°C/120min	96.7	2.3	0.183	0.410	99.0	Mild degradation was observed
Base hydrolysis (5M NaOH)	60min	84.3	10.4	0.087	0.332	99.7	Major degradation
Oxidation(30% H2O2)	85°C/120min	89.0	7.6	0.129	0.370	99.6	Major degradation
Thermal	105°C/120hr	98.8	0.7	0.112	0.423	99.6	No major degradation
Photolytic(White fluorescent light,1.2 million Lux hr and UV light,200 watt-hr/m2	120hr	99.9	Nil	0.161	0.438	99.9	No major degradation
Humidity	90%RH/25°C/120hr	99.4	Nil	0.175	0.440	99.4	No major degradation



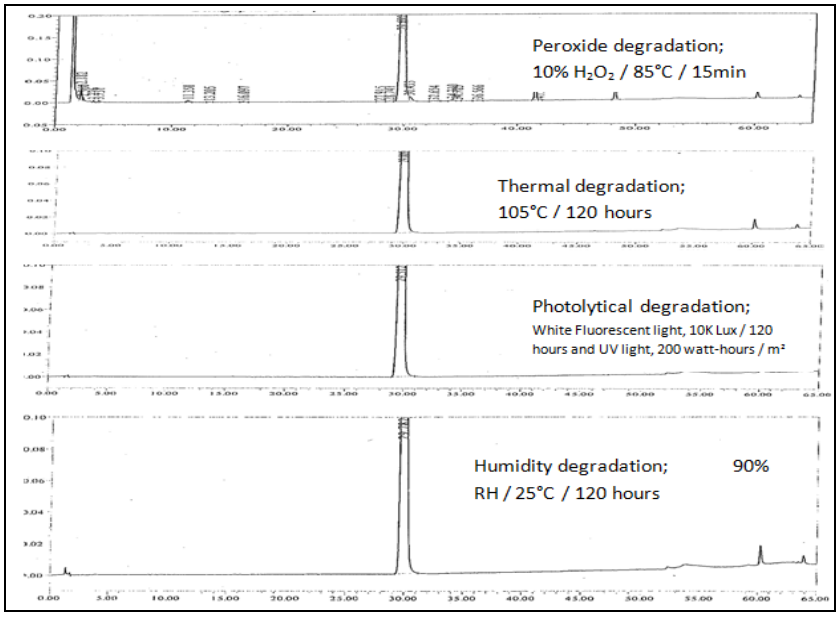


FIG. 6: FORCE DEGRADATION CHROMATOGRAMS OF SITAGLIPTIN

Precision:

The method precision of the related substances method was performed by two analyst by injecting six individual preparations of sitagliptin phosphate with test concentration spiked with 0.15% level of impurities on different days, different columns and on different instruments.

The areas of each impurity obtained from precision experiment from analyst were subjected to statistical evaluation. The results of analyst I, analyst II and over all statistical data are tabulated **Table 4.**

TABLE 4: METHOD PRECISION STATISTICAL DATA

(Analyst-I) (n=6)

Name	Mean(%w/w)	SD	% RSD	95 % confidence Interval(±)
Impurity-1	0.151	0.001	0.7	0.001
Impurity-2	0.148	0.001	0.7	0.001
Impurity-3	0.153	0.001	0.7	0.001

(Analyst-II) (n=6)

Impurity-1	0.152	0.001	0.7	0.001
Impurity-2	0.147	0.001	0.7	0.001
Impurity-3	0.149	0.001	0.7	0.001

Overall statistical data(n=12)

Impurity-1	0.152	0.001	0.7	0.001
Impurity-2	0.148	0.001	0.7	0.001
Impurity-3	0.151	0.001	0.7	0.001

(Analyst-I) (n=6)

LOD and LOQ:

The LOD of the imp-1, imp-2 and imp-3 were 0.011, 0.003 and 0.003 and the LOQ of the imp-1, imp-2 and imp-3 were 0.033, 0.009 and 0.009 respectively (of the analyte concentration is 1500 µg/mL) were established from linearity experiments. Finally the LOD and LOQ values were confirmed by injecting six individual preparations imp-1, imp-2 and imp-3 at proposed LOQ and LOD levels and calculated percentage RSD. The results were presented in **Table 5a** and **5b**.

TABLE 5a: LOQ PRECISION

Preparation	Imp – 1 Area	Imp – 2 Area	Imp – 3 Area
Conc.(% w/w)	0.033	0.009	0.009
1	5129	4784	5211
2	5087	4932	5111
3	5063	4981	5108
4	5008	4872	5057
5	5027	4975	5242
6	5014	4672	5088
Average	5071	4869	5136
% RSD	1.0	2.5	1.4

TABLE 5b: LOD PRECISION

Preparation	Imp – 1 Area	Imp – 2 Area	Imp – 3 Area
Conc.(% w/w)	0.011	0.003	0.003
1	1691	1216	1346
2	1713	1201	1237
3	1587	1203	1341
4	1686	1350	1342
5	1540	1210	1327
6	1975	1230	1401
Average	1669	1235	1332
% RSD	5.5	4.6	4.0

Linearity: The linearity calibration plot for the related substances method was obtained over the calibration ranges from 0.003% to 0.225% i.e is LOQ to 150% of specification level for the imp-1, imp-2 and imp-3.

The correlation coefficient obtained was greater than 0.999. This result shown that an excellent correlation existed between the peak area and the concentrations of the Imp-1, Imp-2 and Imp-3.

Accuracy: The percentage recovery of impurities in Sitagliptin samples varied from 98.5% to 101.1%. The accuracy of all three impurities were established from LOQ to 150% specification level in Sitagliptin bulk drug sample **Table 6.**

TABLE 6: ACCURACY OF SITAGLIPTIN IMPURITIES DATA

Compound	Level %	Amount Added (% w/w)	Amount Found (% w/w)	% Recovery
Imp - 1	LOQ	0.0332	0.0336	101.1
	50	0.075	0.076	100.9
	100	0.151	0.150	99.6
	150	0.225	0.223	99.1
Imp – 2	LOQ	0.009	0.009	100.0
	50	0.075	0.074	98.2
	100	0.150	0.147	98.2
	150	0.224	0.222	99.1
Imp - 3	LOQ	0.009	0.009	100
	50	0.076	0.075	99.1
	100	0.152	0.153	100.7
	150	0.227	0.226	99.6

Robustness:

In all the deliberate varied chromatographic conditions (flow rate, column temperature, wavelength and composition of organic solvent), the USP Plate count of Sitagliptin greater than 10000 and USP Tailing of Sitagliptin is less than 2.0, the resolution between impurity 2 and impurity 3 was greater than 2.0 illustrating the robustness of the method. Results are tabulated in the **Table7-8.**

TABLE 7: ROBUSTNESS EXPERIMENT VALUES

Parameter	Modification	*Resolution between Imp-2 and Imp-3	** USP tailing for Sitagliptin	** USP plate count for Sitagliptin
As per Method conditions	-	4.6	1.2	33211
% of Organic in mobile phase				
% of Organic in mobile phase A	-2% absolute	4.5	1.3	37233
	+2% absolute	4.7	1.2	26125
% of Organic in Gradient	-2% absolute	4.5	1.2	45158
Composition	+2% absolute	4.8	1.3	27022
Flow				
1.08	-10%	4.6	1.3	36015
1.32	+10%	4.7	1.2	30196
Column Oven Temperature				
20°C	-5°	4.5	1.2	31070
30°C	+5°	4.6	1.2	31967
Wavelength				
207nm	-3nm	4.6	1.2	32629
213nm	+3nm	4.6	1.2	33455

TABLE 8: SITAGLIPTIN RT RATION DURING ROBUSTNESS EXPERIMENT

Parameter	Modification	IMPURITY-1	IMPURITY-2	IMPURITY-3
As per Method conditions	-	0.37	1.60	1.64
% of Organic in mobile phase				
% of Organic in mobile phase A	-2% absolute	0.37	1.50	1.54
	+2% absolute	0.37	1.82	1.87
% of Organic in Gradient	-2% absolute	0.38	1.43	1.47
Composition	+2% absolute	0.39	1.75	1.81
Flow				
1.08	-10%	0.38	1.54	1.58
1.32	+10%	0.36	1.69	1.73
Column Oven Temperature				
20°C	-5°	0.37	1.52	1.56
30°C	+5°	0.36	1.73	1.78
Wavelength				
207nm	-3nm	0.38	1.58	1.62
213nm	+3nm	0.38	1.58	1.62

Solution stability:

The % RSD of has no significant changes when we were observed in the content of imp-1, imp-2 and imp-3 during solution stability experiment when performed using the related substance method. The solution stability experiment data confirms that the sample solution used during the related substance determination were stable for at least 24 h.

Application of the developed HPLC method to stability samples and quality monitoring of Sitagliptin Phosphate:

Accelerated and long-term stability studies are carried out to establish retest period or a shelf life of drug product. Sitagliptin Phosphate samples stored at long-term condition (temp: 25 C ± 2 C, relative humidity 60 ± 5%) and accelerated condition (temp:40 C ± 2C relative humidity 75 ± 5%) were analyzed by using the developed HPLC method for a period of 6 months at different intervals. Also, the quality of sitagliptin Phosphate was monitored during the production of three batches by using the developed HPLC method. The results clearly indicated that the drug was stable under long term and accelerated conditions, and there were no interference of the impurities for sitagliptin Phosphate, which demonstrates that developed HPLC method was stability-indicating and well applied for drug stability studies as well as for quality monitoring of sitagliptin Phosphate drug substances.

CONCLUSION: In this paper a simple validated and well defined specific stability indicating HPLC method for the determination of sitagliptin Phosphate related substances was described, and the behavior of sitagliptin Phosphate drug substances under various stress conditions were studied and presented. All the degradation products and process impurities were well separated from the drug, sitagliptin, which demonstrates that the method is stability indicating. The information presented here could be very useful for quality monitoring of sitagliptin bulk drug samples, and also employed to monitor the quality of the drug substances during stability studies.

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