ORIGINAL RESEARCH PAPER

Volume-7 | Issue-2 | February-2018 | PRINT ISSN No 2277 - 8179

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

IMPURITY PROFILING AND REGULATORY ASPECTS OF SITAGLIPTIN ACTIVE PHARMACEUTICAL INGREDIENT

Pharmaceuticals

P. B. N. Prasad* CDSCO, Sr Nagar, Telangana, India, *Corresponding Author

Dr. K. Satyanarayana R & D, NATCO Pharma, Hyderabad

Dr. G. Krishna Mohan Department of Pharmaceutical Sciences, JNTUH, Hyderabad

ABSTRACT

In the recent year, impurity profiling in any active pharmaceutical ingredient (APIs) has become a paramount criterion in pharmaceutical analysis and quality control, espoused by major regulatory authorities like the United States Food and Drug administration (US FDA), Central Drugs Standard Control Organization (CDSCO). Sitagliptin phosphate (monohydrate), a newer dipeptidyl peptidase-4 (DPP-4) inhibitor, an oral hypoglycemic (anti-diabetic) drug intended to be used in type-II diabetes. In the present study an effort has been made to analyze the related substances present in the Sitagliptin API and was spiked. The study was performed by using High Performance Liquid Chromatography (HPLC) with predetermined chromatography conditions. Two impurities namely Ketoamide and Enamine in various batches procured and were analyzed. The method was validated by using Waters HPLC, Model no. Alliance 2695, with 2998 PDA detector, powered by. Empower- 2 software. The acceptance criteria for resolution was kept between Ketoamide and Enamine impurity from system suitability solution was not less than 2.0, tailing factor was not more than 2.0. And relative standard deviation for three replicate injections of reference solution was less than 1.0%. The difference between the results of each specified and unspecified impurities from the six different preparations of test solution injections is ± 0.02 and for the total impurities ± 0.05. The average result of Ketoamide and Enamine impurity was found not more than 0.15%.

Objective: The objective of the present study was to find out and estimate the potential impurities present in the sitagliptin active pharmaceutical ingredients according to the ICH guidelines, as there is no reports available for regulatory compliance.

KEYWORDS

Introduction:

There are some of the organic compounds remain in minute quantities in the manufacturing process as impurities in the final product of active pharmaceutical ingredient, which are related to other raw materials used in the process of manufacturing or some of them are by product formed during the process. There is dearth or no data available in the literature for quantitation of related substances belonging to sitagliptin. As sitagliptin is a potent antidiabetic molecule, presence of small quantities of impurities or related substances of active drug may alter its antidiabetic activity. a sitagliptin is new potent antidiabetic agent and a lack of literature may be due to under patent protection of sitagliptin.

The present study was to develop a suitable gradient HPLC method for the determination of related substances/impurities present in sitaglitpin. Both the United States Pharmacopoeia USP [1] and EP [2] have not published monographs for this drug substance. However, many analytical methods have been reported in literature on the determination of sitagliptin with combination of other anti diabetic drug like metformin hydrochloride. Mostly the analytical methods are reported based on UV Parag P,et.al, 2011[3], Khan 2011[4], Ramzia El-Bagary,et.al, 2011[5] , Spectroflourimetry Ravi PP, et, al 2011[6] and reverse phase HPLC techniques Shyamala M et. 2011[7], Zeng W, et. 2010 [8]. There are some methods which are used to determine Sitagliptin in human plasma and Urine by LC-MS/MS and GC-MS Zeng W,et.al, 2008[9], Nirogi R,et,al ,2008[10], Ucakturk, Ebru 2013[11], Haak T.2015[12]. But no literature has been reported on determination of Sitagliptin phosphate impurities present in the API or in the drug product.

Therefore, these were selected for few related substances which commonly comes in the pathway of manufacturing process or finished API at the time of release and thus planned to develop the HPLC method and validate for related substances of the sitagliptin.

Materials and Methods :

Selection of Impurities:

The following impurities are used for HPLC method validation adopted for the estimation of related substances in Sitagliptin phosphate (monohydrate) as in Fig 1(a) and Fig 1 (b) and selection of impurities with batch numbers (Table 1) were procured from Nutra Specialties Pvt ltd Nellore, AP.

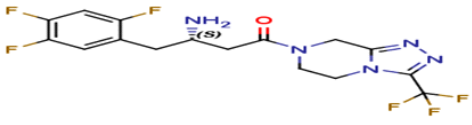


Fig 1 (a) Chemical structure of Ketoamide impurity of sitagliptin

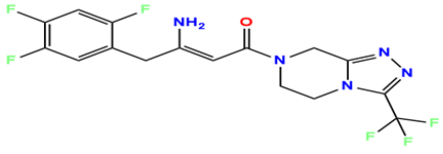


Fig 1(b) chemical structure of Enamine impurity of sitagliptin

Table. 1. Related Substances in Sitagliptin phosphate (Monohydrate)

Name of the impurity	Chemical name	Batch No.
ketoamide impurity	1-[3-(trifluoromethyl)-5,6-dihydro[1, 2,4]triazolo[4,3- a]pyrazin-7(8H)-yl]-4-(2,4,5-trifluorophenyl)butane-l dione	STG-II-331
enamine impurity	(2Z)-4-0xo-4- [3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a] pyrazin-7(8H)-yl]-l- (2,4,5- trifluorophenyl)but-2-en-2-amine.	STG-II-192

Selection of API

The batches of sitagliptin STG-III-23, STG-III-24 and STG –III-25 and also sitagliptin working standard were procured from Nutra Specialties Pvt Ltd Nellore, AP.

Instrument and Chromatographic method conditions for related substances by RPHPLC

Column:Unison-US, C8, 150 X 4.6mm ID, 5µm particle size,

Flow rate: 1.0 ml/min
Wavelength: 210nm
Column temperature: 25°C, Loop 20 µL, Run time 48 minutes,
Diluents: Methanol, Elution: Gradient (as mentioned in Table 4.2.2)

Table. 2. Gradient program:

Time (minutes)	Mobile phase-A (%)	Mobile phase-B (%)
0.01	75	25
14	75	25
32	55	45
40	55	45
40.5	75	25
48	75	25

Buffer preparation: Dissolve 5.22 g of Di potassium hydrogen phosphate in 1500 ml of water and adjust the pH of this solution to 7.2 with ortho-phosphoric acid then filter through 0.45 µ filter paper and Degas. Mobile phase A: buffer: acetonitrile (9: 1) v/v and Mobile phase B: acetonitrile: buffer (7:3) v/v.

Preparation of Sample and standard Solution of impurities on Drug Substance

Impurity standard stock solution preparation (Aml) : Weigh accurately about 5 mg of Ketoamide impurity and transfer it into a 100 ml volumetric flask. Weigh 5mg of enamine impurity and transfer it into same 100 ml volumetric flask. Add about 5 ml of diluent and sonicate to dissolve and made up to the mark with diluent.

System suitability preparation: Weigh accurately about 20 mg Sitagliptin phosphate (monohydrate) working standard into a 10 ml volumetric flask. Add about 5 ml of diluent and sonicated to dissolve and add 0.6 ml of impurity standard stock solution and made up to the mark with diluent.

Ketoamide impurity standard stock solution preparation: Transfer 5mg of Ketoamide impurity into 100 ml volumetric flask, dissolved and diluted to the volume with diluent.

0.15% Ketoamide impurity standard solution preparation: Transfer 0.6 ml of Ketoamide impurity standard stock solution into 10 ml volumetric flask and diluted to the volume with diluent.

Enamine impurity standard stock solution preparation: Transfer 5mg of Enamine impurity into 100 ml volumetric flask, dissolved and diluted to the volume with diluent.

0.15% Enamine impurity standard solution preparation: Transfer 0.6 ml of Enamine impurity standard stock solution into 10 ml volumetric flask and diluted to the volume with diluent.

Sample solution: Weigh accurately about 20 mg of test sample and transfer it into a 10ml volumetric flask, add about 8 ml of diluent and sonicated to dissolve and made up to the mark with diluent, mixed well *Reference solution-A (0.1%) preparation:* Transfer 1 ml of sample solution to 100 ml of volumetric flask and made up to the mark with diluent. Transfer l ml of the above solution to 10 ml of volumetric flask and made up to the mark with diluent.

All impurities spiked test solution: Weigh accurately about 20 mg of Sitagliptin phosphate (Monohydrate) working standard into a 10 ml volumetric flask. Add about 5 ml of diluent and sonicated to dissolve and add 0.6 ml of Aml Impurity stock solution and made up to the mark with diluent.

Calculation:

$$\text{Known impurity \%} = \frac{\text{Known impurity area in test solution X Concentration of reference solution} - A}{\text{Average area of sitagliptin phosphate (monohydrate)in reference solution} - A}$$

$$\text{Maximum unknown individual impurity \%} = \frac{\text{Known impurity area in test solution X Concentration of reference solution} - A}{\text{Average area of sitagliptin phosphate (monohydrate)in reference solution} - A}$$

$$\text{Total unknown impurity} = \frac{\text{Total unknown impurities area in test solution X Concentration of reference solution} - A}{\text{Average area of Sitagliptin phosphate (Monohydrate) in reference solution} - A}$$

Total impurities (%) = Total known impurities+ Total unknown impurities

Method Development

The analytical method of impurities profiling was developed by trials of various ratios of solvent mixture and finally standardized by the gradient programming and other chromatographic conditions with better retention time and good resolution. The trial chromatogram in Fig 2 (a) and Fig 2 (b) and standard chromatograms of sitagliptin and its impurities are represented in Fig 2(c), 2(d), 2(e)

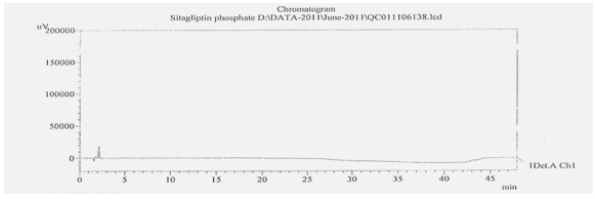


Fig 2(a) Trial Chromatogram

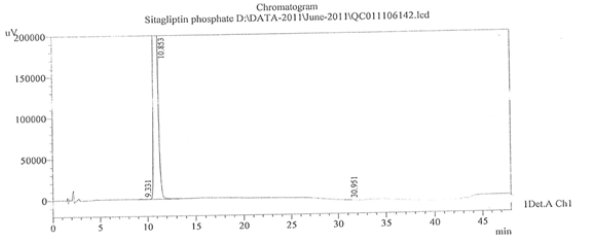


Fig 2 (b) Trial Chromatogram

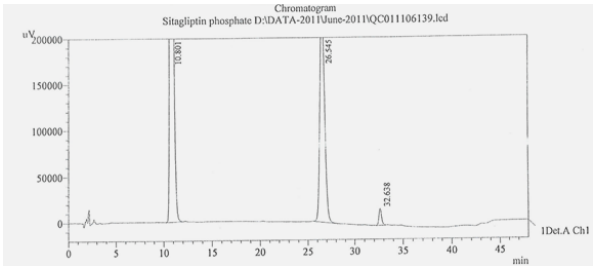


Fig 2(c) standard chromatogram of sitagliptin and impurities of ketoamide and enamine

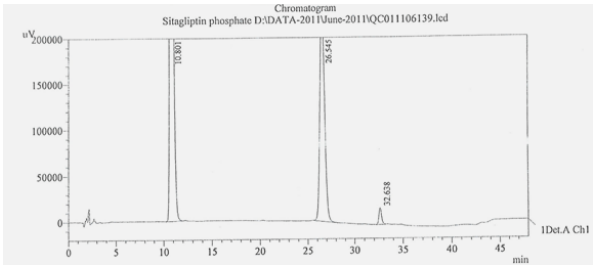


Fig 2 (d) Standard chromatogram of sitagliptin and impurities of ketoamide and enamine

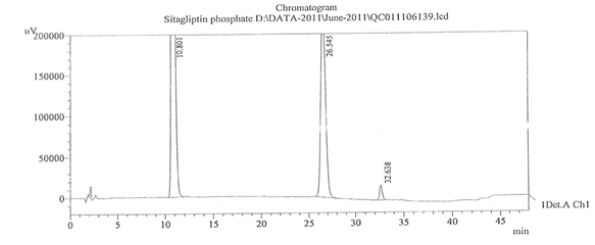


Fig 2 (e) Standard chromatogram of sitagliptin and impurities of ketoamide and enamine

Method validation:

Validation of the developed HPLC method included linearity, range, precision and accuracy, limit of detection (LOD), Limit of quantification (LOQ) and robustness. Method validations is conducted

in accordance with ICH guidelines

Precision:

Inject the solutions to the chromatograph in the following sequential order for sitagliptin impurities (Table 3), order of injections for system precision (Table 4) and order of injections for sample precision(Table 5)

Table. 3. Order of injections for sitagliptin impurity

S.No.	Solution name	No. of injections
1	Diluent as blank	1
2	System suitability solution	1
3	Diluent as blank	1
4	Reference solution-A	3
5	Diluent as blank	1
6	0.15% Ketoamide impurity	1
7	0.15% Enamine impurity	1
8	Diluent as blank	1
9	STG-III-23	1
10	STG-III-24	1
11	STG-III-25	1
12	STG-III-23 +all impurities spiked solution	1
13	STG-III-24 +all impurities spiked solution	1
14	STG-III-25 +all impurities spiked solution	1

Integrate the each known impurity in individual impurity injections and Sitagliptin phosphate (Monohydrate) peak in test solution and all the known peaks based on this, establish the RRT of the individual impurity with respect to Sitagliptin phosphate (Monohydrate) peak in all impurities spiked test solution.

Table. 4. Order of injections for System precision

S.No.	Solution name	No. of injections
1	Diluent as blank	1
2	System suitability solution	1
3	Diluent as blank	1
4	Reference solution-A	3

Table. 5. Order of injections for Sample precision

S. No,	Solution name	No. of injections
1	Diluent as blank	1
2	System suitability solution	1
3	Diluent as blank	1
4	Reference solution-A	3
5	Diluent as blank	1
6	Test solution preparation-01	1
7	Test solution preparation-02	1
8	Test solution preparation-03	1
9	Test solution preparation-04	1
10	Test solution preparation-05	1
11	Test solution preparation-06	1
12	All impurities spiked test solution preparation-0 1	1
13	All impurities spiked test solution preparation-02	1
14	All impurities spiked test solution preparation-03	1
15	All impurities spiked test solution preparation-04-	1
16	All impurities spiked test solution preparation-05	1
17	All impurities spiked test solution preparation-06	1

Integrate any individual unknown impurities based on RRT and calculate the average result of all impurities from test solution by using the formula given under calculation of impurities. Integrate the known peaks based on the RRT in all the injections and calculate relative standard deviation for the area of each individual impurity.

Acceptance criteria as per ICH guidelines and USP:

1.0 Resolution between ketoamide impurity and Enamine impurity from system suitability solution should not be less than 2.0
2.0 Tailing factor of Sitagliptin phosphate peak from system suitability should not be more than 2.0
3.0 Relative standard deviation for three replicate injections of reference solution-A should be less than 1.0%
4.0 The difference between the results of each specified and unspecified impurities from the six different preparations of test solution injections should be ± 0.02 and for the total impurities ± 0.05
5.0 The average result of Ketoamide impurity should not be more than 0.15%
6.0 The average result of Enamine impurity should not be more than 0.15%
7.0 The average result of unspecified impurity should not be more than 0.10%
8.0 The average result of total impurities should not be more than 1.0%
9.0 Relative standard deviation for the area of each individual impurity from six different preparations of all impurities spiked test solution should not be more than 2.0%

$$= \frac{\text{(Average area of each known impurity in LOQ level all imp spiked test solution} - \text{average area of each known impurity in test solution)}}{2a100\text{Average area of each impurity in 100\% authentic solution} \times \text{Concentration of each LOQ level all imp spiked solution}} \times$$

Accuracy and Linearity:

Procedure: Prepare the test solution, system suitability solution, reference solution-A, all impurities standard stock solution and all impurities spiked test solution as mentioned in the procedure under method development and optimization.

Level-l solution: (0.075%) : Transfer 20 mg of the substance to be examined into 10 ml volumetric flask containing 5 ml of diluent, add 0.3 ml of all impurities standard stock solution, dissolved and diluted to volume with diluent.

Level-2 solution: (0.1125%): Similarly add 0.45 ml of all impurities standard stock solution, dissolved and diluted to volume with diluent.

Level-3 solution: (0.15%): Similarly add 0.6 ml of all impurities standard stock solution dissolved and diluted to volume with diluent.

Level-4 solution: (0.1875%): Similarly add 0.75 ml of all impurities standard stock solution dissolved and diluted to volume with diluent.

Level-5 solution: (0.225%): Similarly add 0.9 ml of all impurities standard stock solution dissolved and diluted to volume with diluent.

$$\%\text{Recovery} = \frac{\text{(Average area of each known impurity in impurity spiked test solutions} - \text{average area of each known impurity in test solution)X concentration of standard solution}}{\text{Average area of each impurity in all impurities spiked solution preparation} \times \text{concentration of each known impurity in impurity spiked test solution}} \times 100$$

Relative response factor:

Procedure: Prepare the System suitability solution, Reference solution-A as mentioned in the procedure under method development and optimization.

Sitagliptin phosphate (Monohydrate) standard stock solution preparation: Transfer 5.0 mg of Sitagliptin phosphate (Monohydrate) into 10 ml volumetric flask containing 5 ml of diluents, mixed well and diluted to the volume with diluent.

Ketoamide impurity standard stock solution preparation: Transfer 5.0 mg of Ketoamide impurity into 10 ml volumetric flask containing 5 ml of diluent, mixed well and diluted to the volume with diluent.

Enamine impurity standard stock solution preparation: Transfer 5.0 mg of Enamine impurity into 10 ml volumetric flask containing 5 ml of diluent, mixed well and diluted to the volume with diluent.

RRF stock solution preparation: Transfer 1 ml of each impurity standard stock solution into 10 ml volumetric flask, mixed well and diluted to the volume with diluent.

Level-l RRF solution preparation: (0.075%): Transfer 0.3 ml of RRF stock solution into 10 ml volumetric flask, diluted to the volume with diluent.

Level- 2 RRF solution preparation: (0.1125%): Transfer 0.45 ml of RRF stock solution into 10 ml volumetric flask, diluted to the volume with diluent.

Level- 3 RRF solution preparation: (0.15%): Transfer 0.6 ml of RRF stock solution into 10 ml volumetric flask diluted to the volume with

diluent.

Level-4 RRF solution preparation : (0.1875%): Transfer 0.75 ml of RRF stock solution into 10 ml volumetric flask, diluted to the volume with diluent.

Level-5 RRF solution preparation: (0.225%): Transfer 0.9 ml of RRF stock solution into 10 ml volumetric flask, diluted to the volume with diluent. Inject the solutions to the chromatograph in the following sequential order (Table 6).

Table.6. Order of injections for RRF solution

S. No.	Solution name	No. of injections
1	Diluent as blank	1
2	System suitability solution	1
3	Diluent as blank	1
5	Level-1 RRF solution preparation	3
6	Level-2 RRF solution preparation	3
7	Level-3 RRF solution preparation	3
8	Level-4 RRF solution preparation	3
9	Level-5 RRF solution preparation	3

The known peaks in all the injections are integrated and calculated mean area for each known impurity and Sitagliptin phosphate monohydrate peak at each % level. Correlation Coefficient is calculated from mean area of five different concentrations. The RRF of the individual impurity is established with respect to Sitagliptin phosphate (Monohydrate) Working Standard by using the following formulae at each level. Finally the average RRF value, and RF value for each individual impurity is Calculated and also the %RSD for five RRF values.

Formula:

$$RRF = \frac{AiXWsXPs}{AsXWiXPi}$$

$$RF = \frac{1}{RRF}$$

Ai: Average area of Impurity; As: Average area of Sitagliptin phosphate (Monohydrate) Working standard ; Wi: Impurity weight. Ws: Sitagliptin phosphate (Monohydrate) Working Standard weight ; Ps: Sitagliptin phosphate (Monohydrate) Working Standard Purity ; Pi: Impurity standard purity

Solution stability & mobile phase stability:
Procedure: Prepare the reference solution-A, system suitability solution and test solution as mentioned under materials and methods. Perform the analysis by injecting same test solution stored in ambient condition and the 5°C stored solution and freshly prepared test solution with four different time intervals ("48" Hours).The chromatogram is recorded and the impurities are estimated by using a calculation as given in procedure for estimation of impurities. At each interval, prepared the standard solution freshly, integrated the known peaks in all the injections and calculated the impurities by using the formula given in the procedure.

Statistical analysis:
Results were analyzed using Sigma Plot statistical software (version 11 Copyright © 2008 Systat Software), and were expressed as mean ± standard deviation of the mean (SD). The statistical analysis was carried out using one-way analysis of variance (ANOVA). Statistical P value < 0.05 was considered to be significant.

Results and Discussion:
The method was optimized with the flow rate variation of mobile phase A and B in different ratios in gradient system on column of C_8(150x4.6 mm ID with 5 µm particle size) detection wavelength of 210 nm and retention times of 10.80 min for sitagliptin phosphate, 26.54 min for ketoamide impurity and 32.63 min for enamine. The resolution between ketoamide and enamine is 7.16 and the acceptance criteria in system solubility solution of not more than 2.0 is met (Table 7) and relative standard deviation for sitagliptin with 0.27 and a tailing factor of 1.42 which is within acceptable limits (Table 8)

Table. 7. Resolution between Ketoamide and enamine from system suitability solution.

S.No.	Name	Resolution
1	Ketoamide	---
2	Enamine	7.16
Acceptance criteria	Resolution between ketoamide and enamine in system suitability solution should not be less than 2.	

Table. 8. Relative standard deviation for the area of Sitagliptin Phosphate peak of reference solution –A (n=3)

	Sitagliptin Phosphate
1	80226
2	80662
3	80413
Average.	80434
Standard deviation	218.7
% RSD	0.27.
Tailing factor	1.42

Relative retention time (RRT)
The RRT was determined by injecting the individual solutions of impurities (Ketoamide and Enamine) and spiked test solution. This was used to identify the peak corresponding to the drug and impurities (in Table 9)

Table. 9 Relative retention times

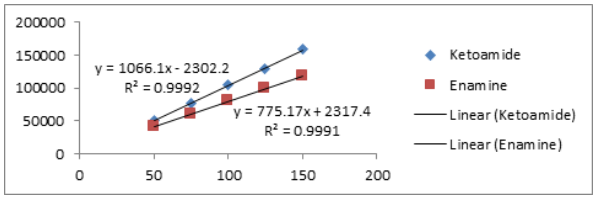
Name of the impurity	Batch No.	Retention time for individual injection	Retention time for Spiked test solution	RRT
Sitagliptin Phosphate	STG-III-23	9.36	9.35	1
Sitagliptin Phosphate	STG-III-24	9.36	9.37	1
Sitagliptin Phosphate	STG-III-25	9.35	9.38	1
Ketoamide	STG-II-331	25.41	25.4	2.72
Enamine	STG-II-192	31.35	31.37	3.35

Linearity of the Ketoamide and Enamine
The linearity of the method for ketoamide and enamine was determined at different standard concentration levels and plotted the graph between the peak area and normal concentrations of respective drug. The linearity data and linearity graph were shown in Table.10 & Fig.3.

Table.10 Linearity data of Ketoamide and Enamine

Concentration (%)	Ketoamide	Enamine
50	50881	40839
75	77723	59939
100	105402	81422
125	129047	98536
150	158480	118437
Slope	1066.1	775.2
Intercept	-2302.2	2317.4
Correlation coefficient	0.9996	0.9996

Fig.3 Linearity graph of Ketoamide & Enamine



Determination of Limit of Detection and Limit of Quantification:
Limit of Detection (LOD):
Preparation of LOD solution for ketoamide: Taken 0.03 mL of ketoamide stock solution in 10 mL volumetric flask, diluted to the volume with solvent mixture.

Preparation of LOD solution for enamine: Taken 0.03 mL of Enamine stock solution in 10 mL volumetric flask, diluted to the volume with solvent mixture.

The S/N ratio and LOD and LOQ for ketoamide and enamine are shown in table 11 and table 12 respectively

Table. 11 Signal to noise ratio values and Limit of detection results

Sno.	Name of the impurity	signal to noise ratio	Result in (%)
1	Ketoamide	2.75	0.007
2	Enamine	2.75	0.008
Acceptance criteria	The signal to noise ratio for aml impurities should be in between 2.0 and 3.0		

Table. 12 Signal to noise ratio values and Limit of quantification results

S.No.	Name of the impurity	signal to noise ratio	Result in (%)
1	Ketoamide	10.04	0.031
2	Enamine	9.98	0.032
Acceptance criteria	The signal to noise ratio for aml impurities should be in between 9.5 and 10.4		

Limit of Quantification (LOQ): The limit of quantification for the ketoamide and enamine was determined by analyzing the samples of sitagliptin at lower limit of quantification injected for six times into the HPLC and calculated the % RSD & % accuracy of the peak area of respective peaks at LOQ. The data was represented in Table.13 and 14

Table. 13 %RSD for the area of aml impurities from different preparations of LOQ level solutions

Injection no.	Keto amide	Enamine
1	26408	26275
2	26291	26046
3	26720	26097
4	26410	26964
5	27000	26631
6	26959	26942
Average	26631	26493
Standard deviation	305.2	411.5
%RSD	1.15	1.55

Table. 14 % Recovery of Ketoamide and Enamine at LOQ level concentration

		Impurity area in STG-111-23	Impurity area in 100% LOQ solution	Impurity area in 100% LOQ level aml impurities spiked test solution	% Recovery
Ketoamide	Preparation-01	Not detected	27756	27691	102.3
	Preparation-02	Not detected	27584	28946	
	Preparation-03	Not detected	27028	27648	
	Average	Not detected	27456	28095	
Enamine	Preparation-01	Not detected	27548	27843	100.4
	Preparation-02	Not detected	27015	26471	
	Preparation-03	Not detected	27117	27669	
	Average	Not detected	27227	27328	
Acceptance Criteria	The % recovery for the area of ketoamide and enamine should not be less than 85 and should not be more than 120				

Accuracy of Ketoamide and Enamine

The accuracy of the related substances in drug substance was expressed by % recovery. This was done at different concentration levels. The recovery of ketoamide and enamine was represented in Table.15 The recovery was found to be 92.6-95.9% for Enamine and 96.7-100.4% for Ketoamide. The % recovery of these indicates the method was accurate.

The statistical data with respect to residual sum of squares for summary output for ketoamide (Table 16) and for enamine (table 19); ANOVA for ketoamide (Table 17) and for enamine (Table 20) and residual output for ketoamide (Table 18) and for enamine (Table 21) are all within statistical limit.

Table. 15 % Recovery at different concentration levels for Ketoamide & Enamine

% Recovery at different concentration levels								
Name of the Impurity		Impurity area in STG-III-23	Impurity area100 % authentic solution	Level-1 solution	Level-2 solution	Level-3 solution	Level-4 solution	Level-5 solution
Ketoamide	Prepn- 1	0	105694	49265	77408	105586	129897	156622
	Prepn-02	0	104329	51524	77773	104977	129692	159052
	Prepn-03	0	105554	51854	77987	105643	127551	159766
	Average	0	105192	50881	77723	105402	129047	158480
	%Recovery			96.7	98.5	100.2	98.1	100.4
Enamine	Prepn-0 1	0	83891	42293	60039	80963	101578	117008
	Prepn-02	0	85253	42736	59955	80480	96460	119728
	Prepn-03	0	86286	37488	59824	82824	97570	118576
	Average	0	85143	40839	59939	81422	98536	118437
	%Recovery			95.9	93.9	95.6	92.6	92.7
Acceptance Criteria	% Recovery for the area of each impurity should not be less than 85 and should not be more than 120							

Residual sum of squares for ketoamide

Table.16 summary output

Regression Statistics	
Multiple R	0.999557416
R Square	0.999115028
Adjusted R Square	0.998820037
Standard Error	1053.014767
Observations	5

Table.17 ANOVA

	Df	Ss	MS	F	Significance F
Regression	1	3755572685	3755572685	3386.938	1.11763£-05
Residual	3	3326520.3	1108840.1		
Total	4	3758899205			

Table.18 Residual out put

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper95%	Lower 95.0%	Upper 95.0%
Intercept	2317.46756	1412.76756	1.640326452	0.199472	-2178.656903	6813.4569	-2178.6569	6813.456903
X Variable1	775.172	13.3197003	58.19740555	1.12£-05	732.782769	817.56123	732.782769	817.561231

Residual sum of Squares for Enamine

Table.19 Summary Output

Regression Statistics	
Multiple R	0.999193746
R Square	0.998388143
Adjusted R Square	0.997850857
Standard Error	1866.126461
Observations	5

Table.20 ANOVA

	Df	Ss	MS	F	Significance F
Regression	1	6471071069	6471071069	1858.20673	2.74782£-05
Residual	3	10447283.9	3482427.967		
Total	4	6481518353			

Table. 21 Residual output

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	942.6	2503.671372	0.37648711	0.73160504	-7025.199707	89109971	-7025.19971	8910.399707
X Variable1	67835	15736.56005	43.10692201	2.7478£-05	628273.9093	72843 5.424	62827 3.909	72843 5.424

Conclusion

The acceptance criteria were met as per the regulatory guidelines both for Ketoamide impurity and Enamine impurity and assay of sitagliptin phosphate. The method has been validated and found to be precise, accurate, linear and robust. The sample solution was stable up to 48 hours. Based on the above data it may be concluded that this method

can be used for regular analysis for impurity profiling or related substances of active pharmaceutical ingredients of sitagliptin phosphate and limiting the percentage of impurities specifications at the acceptable level for release of finished API as per regulatory guidelines such as ICH, WHO and other regulatory guidelines.

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