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Evaluation of Process Impurities and Degradants of Sitagliptin Phosphate by Validated Stability Indicating RP-LC Method

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A new stability-indicating high-performance liquid chromatographic method has been developed for evaluation of degradants, starting materials and process related impurities during the synthesis of sitagliptin phosphate (SIT-P). Separations were achieved on a RP C_{18} column with linear gradient elution of 0.02 M phosphate buffer at pH 7 and acetonitrile as mobile phase constituents. The flow rate was 1 mL/min and photodiode array detector wavelength was set at 210 nm. Method development was carried in order to elute all starting materials, intermediates during reaction monitoring of stage wise synthesis. Sitagliptin phosphate was subjected for stress conditions like acidic and basic hydrolysis, oxidative, photolytic, neutral and thermal degradation and > 95 % mass balance was achieved, thus ensuring stability indicating capability of the method. Major degradants raised due to acid and base stress were studied by LCMS, identified as Imp-1 (*m/z* 193.0) and Imp-4 (*m/z* 234.20), synthesized and subsequently validated along with all other process related impurities as per ICH with respect to specificity, precision, linearity, LOD, LOQ, accuracy, ruggedness and robustness.

Keywords: Sitagliptin, RP-HPLC, Forced degradation, LC-MS, Validation, Reaction monitoring.

INTRODUCTION

Sitagliptin phosphate (SIT-P) is an inhibitor of the dipeptidyl peptidase-4 (DPP-4) enzyme, administered orally as tablet form and is indicated as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus. 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 phosphate (1:1) monohydrate [1,2]. The empirical formula is $C_{16}H_{15}N_5OF_6{\cdot}H_3PO_4{\cdot}H_2O$ and the molecular weight is 523.32.

Only, few analytical methods were available for the determination of impurities in sitagliptin phosphate. One study was reported about isolation and characterization of degradant impurities of sitagliptin [3] and another study reports about synthetic reaction (synthon) of sitagliptin and its derivatives [4]. Two studies were discussed about simultaneous determination of sitagliptin with simvastatin [5] and with metformin [6] from oral dosage-tablet forms. Bioanalytical studies like simultaneous quantitation of metformin and sitagliptin from mouse and human dried blood spots using LDTD-MS [7] and evaluation of the interaction between sitagliptin and cyclodextrin derivatives by CE-NMR [8] and other studies like determination of sitagliptin in human urine by mass spectrometry [9], simultaneous determination with combination drug products [10] were reported.

Although methods related to degradant impurities of sitagliptin were reported, Literature survey reveals that, no reverse phase LC method available for the evaluation of starting materials, process related impurities along with major degradants of sitagliptin phosphate. Hence, a stability indicating RP-HPLC gradient method was developed and fully validated as per ICH guideline, where all the impurities during process mapping and reaction monitoring at different stages were well resolved. Stability indicating capability of the method was evaluated by stress testing and LC-MS studies were carried to identify possible degradants and evaluated their presence in drug substance and their interference with process related impurities.

EXPERIMENTAL

Working standard, API of sitagliptin phosphate and its impurities with > 98 % were supplied by Dr. Reddy's Laboratories Limited, Hyderabad, India. HPLC grade acetonitrile, ACS grade orthophosphoric acid (> 85 % purity) and Empure grade KH_2PO_4 and KOH pellets were purchased from Merck, Darmstadt, Germany. High purity water was prepared by using Milli Q Plus water purification system (Millipore, Milford, MA, USA).

We used the HPLC system (Waters, Milford, USA) consisting of quarternary solvent system embedded with auto sample manager. Output signal was monitored using and a photodiode array (PDA) detector and data processed using Empower2 software. Cintex digital water bath was used for hydrolysis studies. Photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India). The pH of the solutions was measured by a pH meter (Thermo Orion Model 420 A, USA). All solutions were degassed by ultra-sonication (Power Sonic 420, Labtech, Korea) and filtered through a 0.45-mm Nylon 6,6 filter (PALL life sciences, USA).

Chromatographic conditions: The method was developed using Waters Xterra RP18 250 × 4.6 mm, 5 µ column (Waters, Milford, USA) with a mobile phase containing a gradient mixture of solvent A and B. Prepared 0.02 M phosphate buffer, pH adjusted with diluted KOH solution to 7.0 and mixture of buffer:acetonitrile in 95:5 v/v is used as solvent-A and 80:20 v/v acetonitrile and water is used as solvent-B. 70:30 v/v mixture of buffer and acetonitrile is used as diluent. The flow rate was set at 1.0 mL/min with gradient program (time in min and % B) as 0.01/0.0, 15/50, 40/50, 45/80, 53/100, 54/0.0, 65/0.0. Injection volume was set at 10 µL and column oven temperature at 45 °C. Sitagliptin phosphate and its impurities were monitored with PDA-UV set at 210 nm.

Preparation of standard, test solutions and impurity stock solution: Both test and working standard solutions of sitagliptin phosphate were prepared by dissolving 15 mg of respective working standard and API (bulk drug) in diluent to meet final concentration of 1.5 mg/mL.

Primary impurity stocks solutions were prepared by dissolving appropriate amounts of each impurity separately in diluent and pooled impurity stock was prepared by further diluting each primary stock with diluent.

Forced degradation and LC-MS studies for identification of degradants: The specificity of the developed LC method sitagliptin phosphate was carried out in the presence of their impurities. Sitagliptin phosphate drug substance at an initial concentration of 1500 µg/mL was subjected to expose for chemical stress condition of acid (0.5 N HCl at 70 °C), base (0.1 N NaOH at room temperature), hydrolytic (70 °C), oxidation (10 % H_2O_2 at room temperature) and physical stress at UV light as per ICH (1.2 M Lux-hours), heat (105 °C), to evaluate the ability of the proposed method to separate respective active from their degradation products. For heat and light studies, study period was continued till 10 days whereas, for hydrolytic, acid, base and oxidation was below 24 h. Peak purity test was carried out for main peak as well as major degradents by PDA detector. Assay of stressed samples was performed (at 100 µg/mL) by comparison with qualified reference standards and the mass balance (% assay, % degradation products) was calculated.

LC-MS study for identification of degradants: LC-MS/MS system (Agilent 1200 series liquid chromatograph coupled with Applied Biosystems 4000 Q Trap triple quadrupole mass spectrometer with Analyst 1.4 software, MDSSCIEX, USA) was used for identification of degradant formed during forced degradation studies. LCMS Grade salts and solvents (Merck, Germany) were used for the analysis. 0.01 M ammonium acetate, pH adjusted to 7.0 with diluted ammonia is selected as buffer keeping rest of chromatographic conditions same as mentioned. The analysis was performed in positive electro-spray-positive ionization mode, the ion source voltage was 5000 V and the source temperature was 450 °C. GS1 and GS2 were optimized to 30 and 35 psi, respectively. The curtain gas flow was 20 psi.

Method validation: The proposed method was validated as per ICH guidelines [12-14].

Precision: Repeatability was assessed by injecting six individual test preparations as per method in to LC system, which were prepared by spiking accurate volumes of all impurity pooled stock solution to meet 0.15 % of target concentration of SIT Phosphate. *i.e*., 2.25 µg/mL of 1500 µg/mL. The % mean and % RSD of each impurity was evaluated.

As a part of ruggedness, intermediate precision of the method was also evaluated by different analyst, different HPLC instrument with different column and performing the analysis on different day.

Limit of detection (LOD) and limit of quantitation (LOQ): The LOD and LOQ for all impurities along with sitagliptin phosphate were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. A precision study was also carried out at the LOQ level by injecting six individual preparations and calculating the RSD (%) of the area.

Linearity: Linearity test method was performed by preparing stock solutions followed by further dilutions of all impurities including sitagliptin phosphate at six concentration levels ranging from LOQ to 150 % of target concentration. Peak area *versus* concentration data was treated by least-squares linear regression analysis and slope, y-intercept, coefficient of correlation, bias at 100 % were evaluated.

Accuracy: The accuracy of the test method for all the impurities was evaluated in triplicate preparations of test sample spiked with all impurities at each level ranging from 50 % to 150 % and recovery at LOQ was performed by preparing six test solutions spiked with all impurities. % Added *vs*. % found and % recovery for each impurity was evaluated.

Robustness: To determine the robustness of the developed method, experimental conditions were deliberately altered and resolution, tailing factors and RRT's of all impurities were monitored. ± 0.2 mL/min of flow rate, ± 5 °C of column temperature, ± 10 % of organic modifier in mobile phase-B (acetonitrile) and ± 0.2 Units of pH of the mobile phase were altered from original conditions.

Solution stability and mobile phase stability: Solution stability was determined by leaving solutions of standard and spiked test sample in tightly capped volumetric flasks for a period of 48 h at room temperature as well as refrigerator (2-8 °C) during which they were assayed at 24 h intervals. Mobile phase prepared during beginning of the study period and not changed during the experiment and its stability was determined by analysis of freshly prepared sample solutions at 24 h intervals till 3 days and the results were compared with those obtained from freshly prepared reference standard solutions.

RESULTS AND DISCUSSION

Method development and optimization: The main aim of the method development is to separate all reaction related process and degradant impurities of sitagliptin phosphate (Fig. 1). As the pKa of sitagliptin phosphate (strongest base) is at 8.78 and its UV maxima was observed at 210 nm, initial method optimization trials were carried choosing phosphate buffers and acetonitrile whose UV cutt-off is < 205 nm, as mobile phase constituents. Impurity blend was prepared by spiking all impurities at 0.1 % of initial target concentration of test sample (1.5 mg/mL) and used to assess separation efficiency. Trials were performed with different pH and the promising separations were noticed at 6 to 8 pH. Using YMC basic C18 150 × 4.6 mm, 5 µ column at pH 7.5 phosphate buffer with a linear gradient, Imp-5 and 6 were completely merged and asymmetric peak shape observed for IMP-4 and 5. Using Betasil C-18 250 mm × 4.6mm, 5.0 µm column, resolution was < 1.2 for Imp-1 and 2, even resulted in gradient artefacts. With symmetry C18 150 × 4.6 × 3.5 µ column, resolution was < 1.5 for Imp-9 and 10 and split pattern for main peak was observed. To improve resolution, 0.1 % octane sulphonic acid was implemented in mobile phase as ion-pair, resulted in decreased sensitivity and s/n ratio. Linear step gradient with × terra RP-18 250 mm × 4.6 mm, 5.0 µm column, with mobile phase A at pH 7.0 and acetonitrile and water in 8:2 v/v ratio as B, showed optimistic separation of all impurities related to sitagliptin phosphate. During reaction monitoring, solvent like toluene and STP-3A impurity and its diastereomer (Fig. 4) were also well separated along with rest of impurities. Upon stress studies, major and minor degradants were well separated from each other at column temperature set with 45 °C and gradient of time in min and %B) as 0.01/0.0, 15/50, 40/50, 45/80, 53/100, 54/0.0, 65/0.0. During day to day variation of

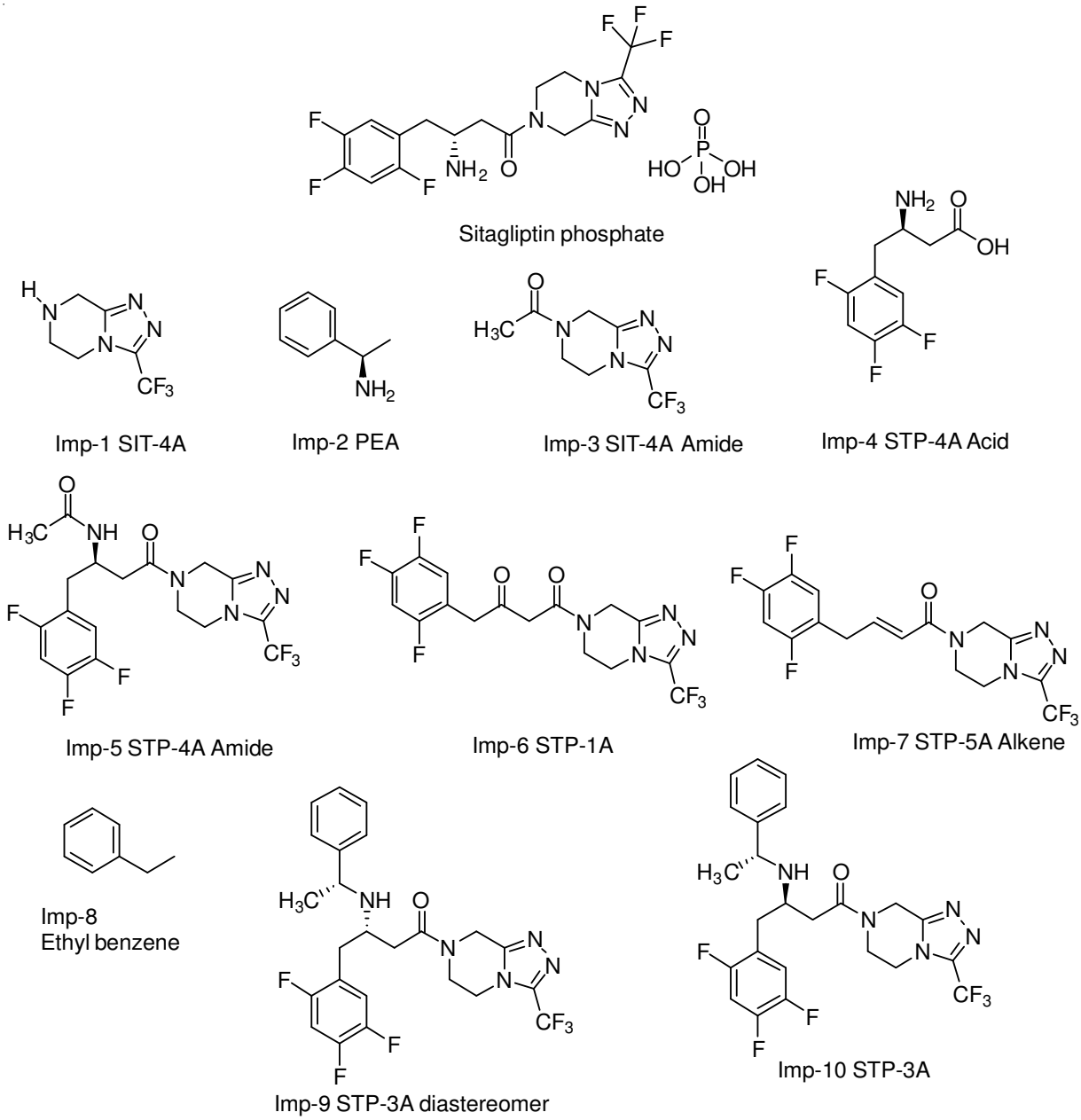

Fig. 1. Sitagliptin phosphate and its impurities (degradants and process related)

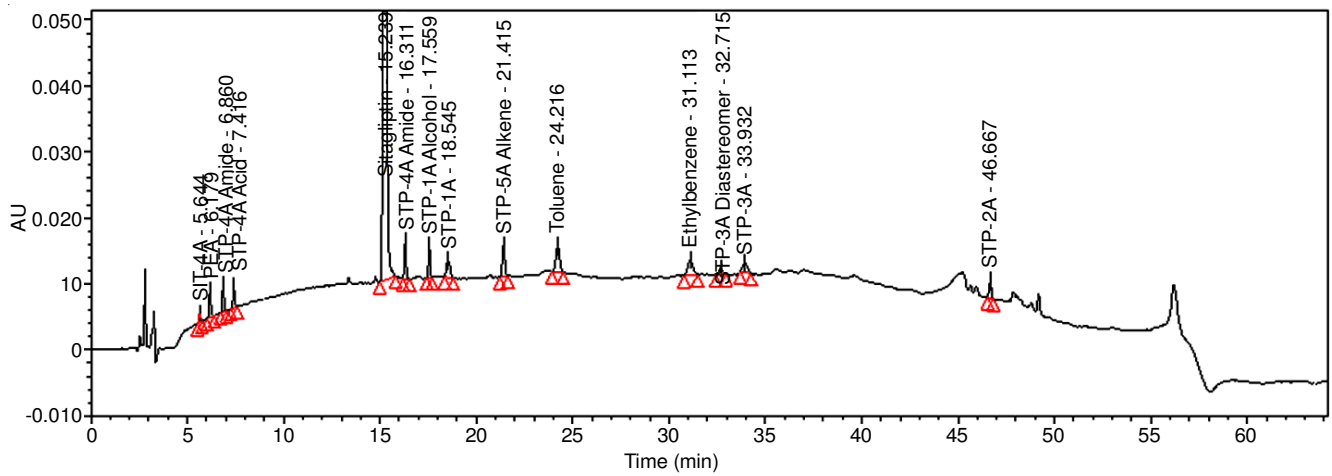

Fig. 2. All impurity spiked chromatogram

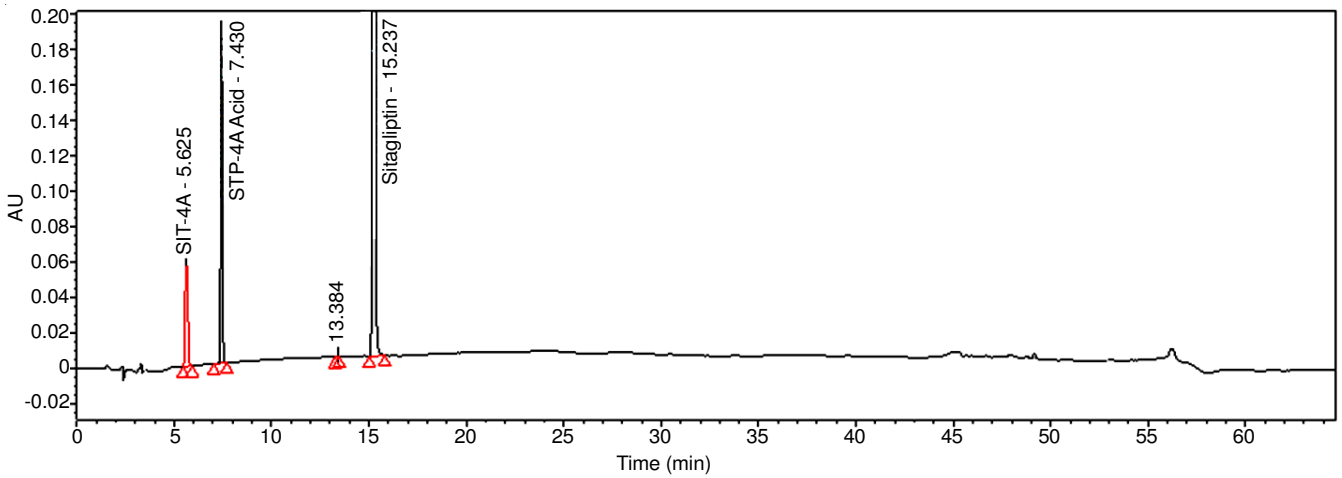

Fig. 3. Acid stress sample chromatogram

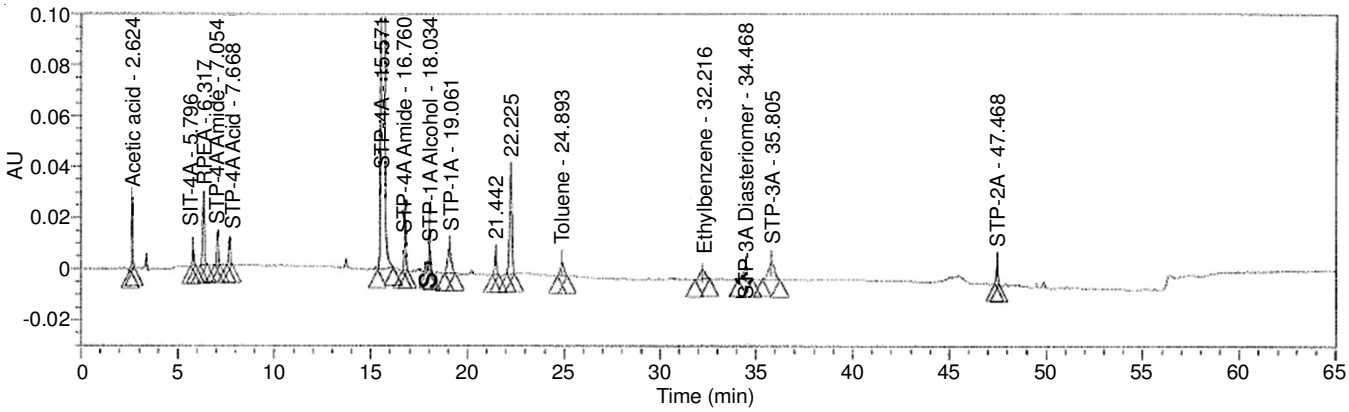

Fig. 4. Chromatogram of sample during reaction monitoring

mobile phase-A stability, turbidity was observed and addition of 5 % acetonitrile to buffer resulted in increased stability. Satisfactory S/N ratio with > 10 was attained for all impurities at LOQ < 0.04 % of target concentration *i.e.* 1.5 mg/mL. With finalized chromatographic conditions, choosing volatile mobile phase like ammonium acetate at 0.01 M pH 7.0, mass compatible method also showed satisfactory separation and *m/z* for degradants.

Outcome of forced degradation and LC-MS studies: Significant degradation of sitagliptin phosphate was achieved by acid and base hydrolysis leading to formation of Imp-1 (*m/z* 193.0) and Imp-4 (*m/z* 234.20) and was confirmed by LCMS studies (Fig. 5). Few unknown impurities < 0.5 % raised due to peroxide stress, were identified by MS with *m/z* 207.30 at RRT 0.36, *m/z* 424.20 at RRT 1.19 and *m/z* 453.50 at RRT 0.81 (Fig. 6). Mild degradation < 0.3 % was observed in water hydrolysis, thermal and photolytic (light) degradation. Spectral purity was assessed by photo diode array, where peak purity and threshold were evaluated for all stressed samples spiked with all process related impurities, in order to observe any interferences. Mass balance (% assay, net degradation and total degradation) was performed by diluting stressed at assay

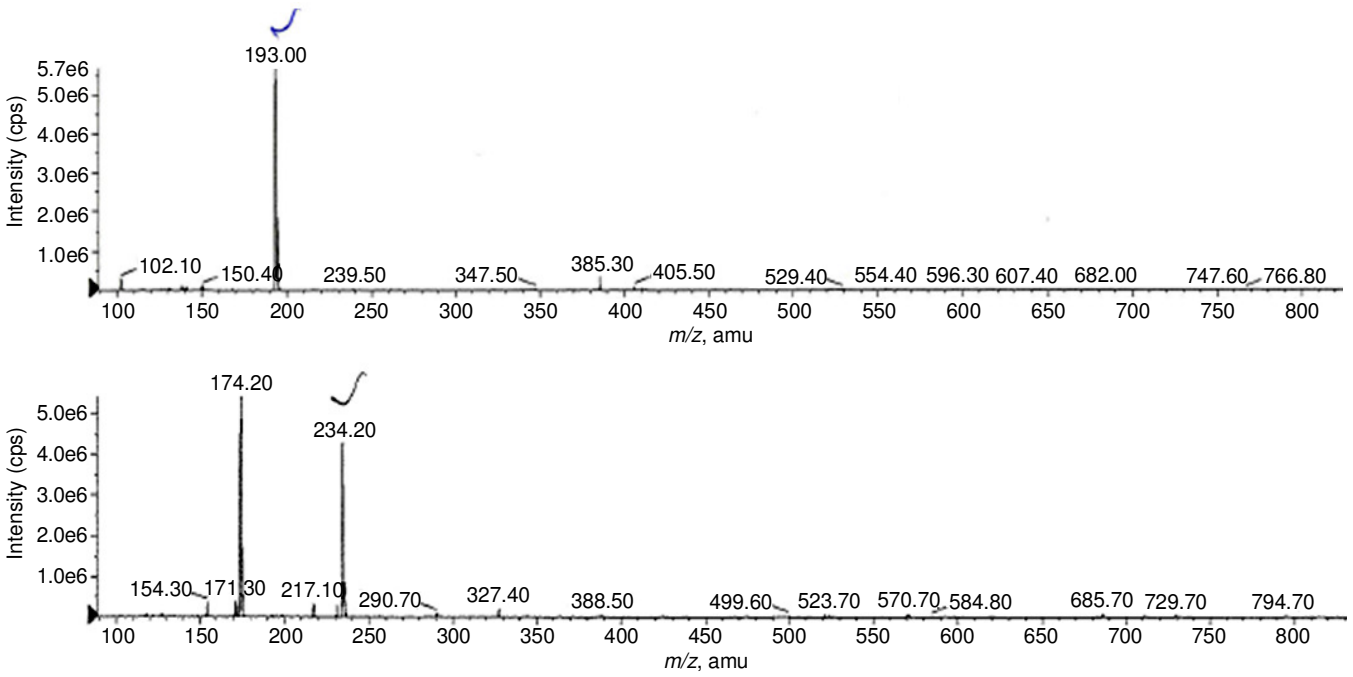

Fig. 5. LC-MS chromatogram of acid stress sample (*m/z* 193 : SIT-4A, *m/z* 234 : STP-4A acid impurities)

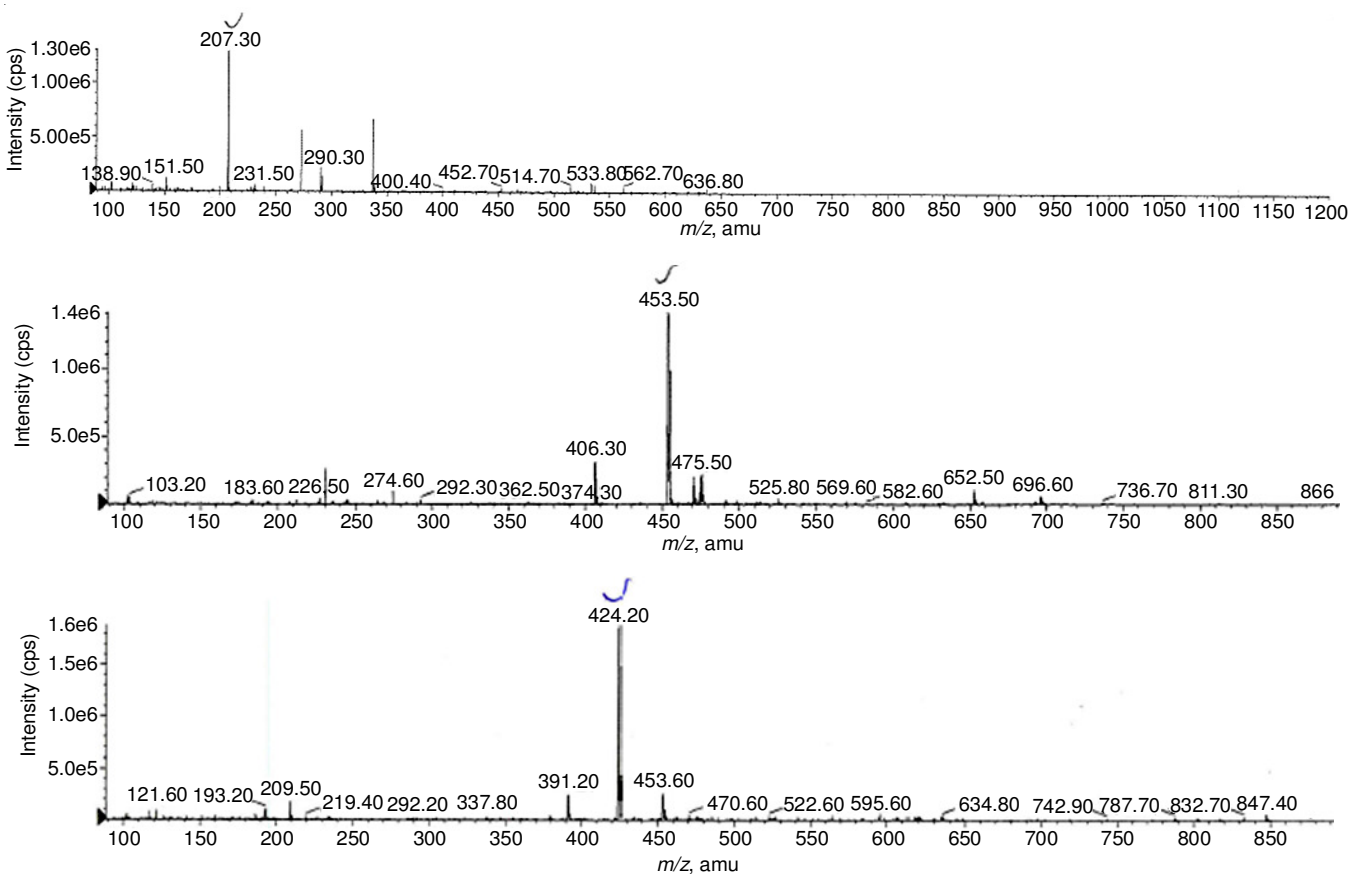

Fig. 6. LC-MS chromatogram of peroxide stress sample (*m/z* 207, *m/z* 453, *m/z* 424 corresponds to unknown)

concentration of 100 µg/mL and calculated against reference standard. The purity and assay of sitagliptin phosphate was found unaffected by the presence of its impurities and degradation products and thus confirms the stability-indicating power of the method (Table-1).

Outcome of stability studies and reaction monitoring: Stability studies for sitagliptin phosphate in primary packing (PE bag, along with silica gel) starting from initial to 6 months accelerated condition 40 °C/75 %RH were analyzed and impurity trending was evaluated. No potential degradants were observed

TABLE-1
FORCED DEGRADATION STUDIES

Sample name	Stress condition	Spectral purity**				
		Degradation (%)	Angle	Threshold	Flag	Mass balance* (%)
Unstressed	NA	0.03	0.094	0.268	No	99.9
Acid stressed	0.5 N HCl 70 °C 18 h	13.45	0.112	0.297	No	98.2
Base stressed	0.1 N NaOH RT – 17 h	5.18	0.318	0.366	No	97.4
Oxidation stressed	10 % H_2O_2 RT 48 h	2.27	0.065	0.382	No	97.1
Water stressed	70 °C 48 h	0.34	0.103	0.367	No	99.3
Thermal stressed	105 °C 3 days	0.18	0.115	0.305	No	100.2
Photo stressed	1.2 M Lux	0.09	0.125	0.293	No	99.7
Photo stressed	Visible region	0.09	0.092	0.309	No	99.1

*Mass Balance = % assay + % impurities + % sum of all degradants; **As per Empower software: Purity angle should be less than purity threshold with no flag.

greater than or equal to identification threshold and rest of known impurities were monitored. During reaction monitoring, STP-1A alcohol and STP-2 impurity was identified (Fig. 4) at retention time of 17.6 and 47.5 min and were controlled in further stages of synt-process. Hence these impurities are not included in specification and were not validated.

Method validation

Precision: The % RSD for the peak areas of all the impurities in method precision and intermediate study was < 2.2 %. Table-2 demonstrated that the method is precise and stood rugged, resisting day to day, system to system, column to column and analyst to analyst variations (Table-2).

LOD and LOQ: S/N ratio with > 10 for LOQ ranging from 0.012 to 0.033 % and S/N ratio with > 3 for LOD ranging from 0.004 to 0.011 % of test concentration was achieved. Mean values of precision at LOQ of all impurities including sitagliptin phosphate reported in Table-3 and recoveries at LOQ level to 150 % level are in the range of 90.8 to 111.2 %. This shows the method's extraction efficiency and sensitivity towards recovery and sensitivity.

Linearity: Linear calibration plot for the related substance method was obtained over the calibration ranges tested, *i.e.*, LOQ to 150 % of the specification level. The correlation coefficient obtained was greater than 0.999 for all the components. The slope and y-intercept and bias values are also provided in Table-4, which confirmed good linearity between peak areas and concentration.

Accuracy and range: Recovery of impurities in spiked studies ranged from 91.4 to 107.9 % and this satisfactorly fulfills

TABLE-2
PRECISION AND INTERMEDIATE PRECISION

Name	Precision			Intermediate precision		
	RRT's	Impurity (%)	RSD (%)	RRT's	Impurity (%)	RSD (%)
IMP-1	0.38	0.168	1.8	0.37	0.173	1.8
IMP-2	0.41	0.157	1.4	0.41	0.166	1.2
IMP-3	0.46	0.151	1.1	0.45	0.157	0.7
IMP-4	0.49	0.166	2.0	0.49	0.178	1.2
IMP-5	1.07	0.154	1.1	1.09	0.157	0.5
IMP-6	1.21	0.152	1.6	1.24	0.158	0.8
IMP-7	1.40	0.170	1.1	1.42	0.169	0.5
IMP-8	2.04	0.143	1.2	1.97	0.131	1.4
IMP-9	2.13	0.144	0.8	2.07	0.139	0.7
IMP-10	2.21	0.151	1.7	2.15	0.157	1.7

RRT's must be comparable, % RSD for precision and inter-precision NMT 10 %.

TABLE-3
LOD, LOQ, ACCURACY

Name	LOD	Mean precision at LOQ	Recovery (%)			
			LOQ	50 %	100 %	150 %
IMP-1	0.010	0.032	102.1	93.0	99.4	99.5
IMP-2	0.009	0.014	95.4	105.4	104.7	107.9
IMP-3	0.004	0.012	111.2	102.7	101	103.6
IMP-4	0.006	0.017	102.6	94.6	100.5	100.2
IMP-5	0.004	0.012	90.8	107.7	105.1	106.7
IMP-6	0.006	0.016	106.9	103.4	103.7	105.7
IMP-7	0.004	0.012	99.5	107.2	104.5	107.1
IMP-8	0.005	0.015	95.1	97.2	96.6	95.6
IMP-9	0.011	0.033	104.3	91.4	91.4	93.2
IMP-10	0.011	0.032	111.1	94.9	100.4	103.0
SIT-P	0.006	0.014	100.2	98.9	100.4	101.1

Obtained USP s/n = 3 for % LOD and 10 for % LOQ. % RSD for LOQ Precision is NMT 15 %; Recovery at LOQ should be within 85-115 %.

TABLE-4
LINEARITY AND REGRESSION

Name	Slope	y-Intercept	Correlation coefficient	Bias at 100 %
IMP-1	4914074	-191.88	0.9998	-1.75
IMP-2	15809697	-128.07	0.9998	-0.36
IMP-3	17055188	33.48	0.9998	0.09
IMP-4	10332294	151.15	0.9999	0.65
IMP-5	19792711	-297.98	0.9999	-0.68
IMP-6	20220996	-599.23	0.9999	-1.34
IMP-7	30256445	-3.25	0.9999	-0.01
IMP-8	38974374	-3172.45	0.9992	-3.69
IMP-9	16298916	826.11	0.9998	2.08
IMP-10	19466903	-860.91	0.9997	-2.00
SIT-P	14573611	740.93	0.9999	0.67

For linearity: Correlation coefficient > 0.997 and bias should be < ± 5.0 %.

the recovery of all known impurities (process and degradatns). Refer to results presented in Table-3. Range of the method for all impurities were verified by performing precision at lower limit *i.e*., LOQ and higher limit *i.e*., 150 % of target concentration and results found satisfactory.

Solution stability, mobile phase stability: Mobile phase was found stable till 3 days, when compared the results with freshly prepared spiked sample at different time intervals *versus* initial day results. During bench top solution stability, Imp-1 and 4 were enhanced leading to failure of acceptance criteria as per ICH. Hence stability study was performed hourly basis and found the solutions were stable till 18 h at bench top. Standard and spiked sample found stable at refrigerator condition till 3 days.

Robustness: In all the varied chromatographic conditions (flow rate, pH, column temperature and composition of organic solvent), the resolution between all pairs of compounds was within acceptable limits (> 2) and tailing factor for sitagliptin phosphate and its impurities was less than 1.3.

Conclusion

The simple gradient reversed phase LC method developed for quantitative analysis of sitagliptin phosphate (SIT-P) and its 10 impurities in drug substance was found to be precise, accurate, linear, robust and specific. Satisfactory results were obtained from validation of the method. The method is stability-indicating and can be used for routine analysis of reaction monitoring, stability and bulk production samples.

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