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A New Stability Indicating Reverse Phase HPLC Method for the Determination of Related Substances of DPP4 Inhibitor Drug: Linagliptin

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A simple, sensitive, selective and reproducible stability indicating reverse phase HPLC method has been developed and validated for the determination of related substances of linagliptin drug substance. Separation of impurities was carried out on a YMC Pack ODS-AQ, 5 µm (250 × 4.6 mm) column. Column eluent consists of gradient mixing of phosphate buffer at pH-3.7 as mobile phase A and a mixture of acetonitrile, methanol and water in the ratio of 600:250:150 v/v/v as mobile phase-B. Gradient elution at a flow rate of 1.0 mL/min and injection volume is 10 µL. The UV/vis detector is set to a wavelength of 226 nm and the column oven is set at 30 ºC. The detector response was found to be linear over the concentration range of 0.07-1.1 µg/mL with correlation coefficients greater than 0.999 for linagliptin and its related substances. LOD values for specified impurities range from 0.004-0.005% w/w and LOQ values from 0.013-0.015% w/w. The accuracy obtained by the proposed method was found to be in the range of 93.2-101.3%. Degraded and process impurities are well separated and the method has been validated for specificity, precision, ruggedness, linearity, accuracy and robustness as per ICH guidelines.

Keywords: Linagliptin, Related substances, HPLC, Dipeptidyl peptidase-4.

INTRODUCTION

Linagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor drug and identified chemically as 8-[(3*R*)-3-amino-1-piperidinyl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl)-methyl]-3,7-dihydro-1*H*-purine-2,6-dione (m.f. $C_{25}H_{28}N_8O_2$; *m.w.*: 472.55) [1]. Linagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor class medication used for the treatment of hyper-glycemia in type-2 diabetes. Dipeptidyl peptidase-4 enzymes were degrading the incretin hormones called glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are controls the blood glucose levels in the human body. Linagliptin suppress the degradation of incretin hormones and stimulate the pancreas to produce insulin, ultimately control of diabetes mellitus type 2. Insulin act to absorb the glucose from the blood into the tissues, so the body can use the glucose to produce energy and keep blood glucose levels stable. DPP-4 inhibitors are used along with diet and physical exercise to lower blood glucose in adults with type 2 diabetes. Uncontrolled, blood glucose can lead to severe problems, including blindness, nerve and kidney damage and heart disease.

Related substances present in the drug substances and drug products can impact the quality, safety and efficacy of the drug substance and drug product. It is important to quantify, identify and characterize the related substances in drug substances and drug products. Related substances may originate through various phases in the route of synthesis or storage of drugs and drug products. Related substances that emerged during the linagliptin process development were identified prepared and characterized [1]. Process and raw material related impurities, linagliptin enantiomer, linagliptin regioisomer, diquinazolinyl linagliptin, reverse linagliptin, dimethylamino linagliptin, bromobutene linagliptin are proposed, based on the route of synthesis and structures of impurities shown in Fig. 1. Linagliptin enantiomer (impurity-I) and linagliptin regioisomer (impurity-II) are raw material related impurities, other impurities are process related impurities.

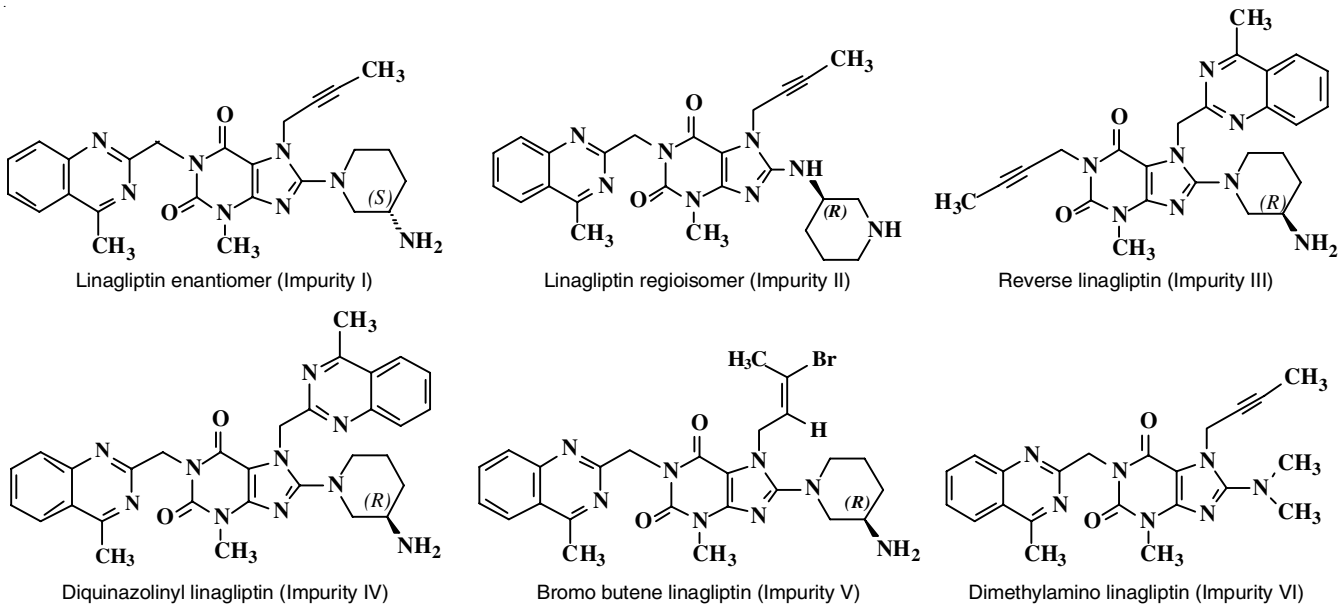

Fig. 1. Chemical structures of related substances of linagliptin

In literature, various techniques have been reported for the quantification of lingliptin in API, drug products and plasma samples [2-10]. Few methods have been reported for related substances for the drug products dose combination with linagliptin drug substance [11-15]. The aim of this study is to develop a reproducible, sensitive and cost effective method for the determination of process and degraded impurities in linagliptin drug substance by RP-HPLC.

EXPERIMENTAL

Linagliptin and its related substances were procured from APL Research Centre-II (A Division of Aurobindo Pharma Ltd). Potassium dihydrogen phosphate, orthophosphoric acid, HPLC grade acetonitrile and methanol were purchased from Merck India. Milli-Q grade (Millipore, USA) water was used during experiments. PVDF membrane filters having pore size of 0.45 µm were procured from Merck Life Science Pvt. Ltd.

All experiments were carried out on a Waters alliance e2695 (Waters Corporation, Milford, USA), separation module consisting of a binary pump, column oven and an auto-injector equipped with 2489 UV/visible detector. Peak purity was performed on 2998 photodiode array detector (PDA). Empower software was used for signal monitoring and data processing.

Preparation of sample solutions and buffer: System suitability solution was prepared by dissolving about 0.5 mg/mL of linagliptin sample enriched with linagliptin regioisomer and reverse linagliptin in the diluent. A mixture of water and acetonitrile in the ratio of 80:20 v/v was used as diluent. Sample solution at about 0.5 mg/mL concentration is prepared by adding 5 mL of acetonitrile to 25 mg of sample and sonicate for 2 min, then add 30 mL of diluent sonicate to dissolve and then make upto 50 mL with diluent. Standard solution prepared at a level of 0.75 µg/mL by diluting the stock solution containing 0.5 mg/mL of linagliptin working standard.

Buffer solution of 20 mM potassium dihydrogen phosphate was prepared by dissolving 2.72 g/L of respective salt in water and pH adjusted to 3.7 ± 0.05 with orthophosphoric acid. Buffer solution filtered through 0.45 µm porosity membrane filter. Solvent mixture prepared by mixing acetonitrile, methanol and water in the ratio of 600:250:150 v/v/v.

RESULTS AND DISCUSSION

Method development: The related substances method of a drug substance should separate all possible process related impurities, degradation impurities, raw materials and intermediates from each other and from the active pharmaceutical ingredient. The linagliptin enantiomer impurity was determined by the chiral method by using a chiral column [16]. During method development studies, different column stationary phases and distinct pH values of the mobile phase were studied. Different solvent compositions (acetonitrile and methanol) were used for better separation between impurities and linagliptin. The wavelength selected for the monitoring of linagliptin and its impurities is 226 nm for better sensitivity.

Method development was initiated by using KH_2PO_4 buffer (0.02 M, pH adjusted to 2.5 with OPA) as mobile phase-A and acetonitrile as mobile phase-B with the column oven at 25 ºC. The gradient programme (time in min/% mobile phase-B) 0.01/10, 15/30, 30/60, 40/80 was applied on the Sunfire C_{18} column with dimensions of 250 × 4.6 mm and 5 µm. Reverse linagliptin (impurity-IV) is very closely eluted to linagliptin. Linagliptin regioisomer (impurity-II), linagliptin and reverse linagliptin (impurity-III) are very closely eluted on octyl silane (C_8) stationary phase. Trials were carried out on different make C_{18} columns. Symmetry C_{18}, X-Bridge C_{18}, Inertsil ODS-4, Eclipse XDB C18 and YMC Pack ODS-AQ. Among all the columns, optimal separation between all the peaks was achieved on the YMC Pack ODS-AQ column. The effect of pH of phosphate buffer in the range between 2 and 6 was studied on separation. Optimal separation was achieved between linagliptin and its related substances with 0.02 M aqueous KH_2PO_4 at pH 3.7. For mobile phase-B, a mixture of acetonitrile, methanol and water in the

ratio of 600:250:150 v/v/v was chosen from 750:150:150, 750:200:100, 700:200:100 and 600:300:100 v/v/v. The effect of column oven temperature was investigated in the temperature range of 25 to 40 ºC, and 30 ºC chosen as the column oven temperature.

Detection method: Separation of linagliptin and its related substances was achieved on the YMC Pack ODS-AQ column having dimensions of 250 × 4.6 mm and 5 µm; with 0.02 M KH_2PO_4, pH 3.7 buffer as mobile phase-A and a solvent mixture of acetonitrile, methanol and water in the ratio of 600:250:150 v/v/v as mobile phase-B at a flow rate of 1.0 mL/min with gradient elution. The gradient programme applied for the separation is (T_{min}/% Mobile phase-B) 0.01/25, 30/40, 45/80, 55/80, 57/25, 65/25. The column compartment was thermostated at 30 ºC and the analysis was monitored at 226 nm by injecting 10 µL of sample solution. The retention time of linagliptin is about 24 min. The elution order of linagliptin and its related substances was linagliptin regioisomer (impurity II), linagliptin, reverse linagliptin (impurity III), diquinazolinyl linagliptin (impurity IV), bromo butene linagliptin (impurity V) and dimethylamino linagliptin (impurity VI), respectively and shown in Fig. 2.

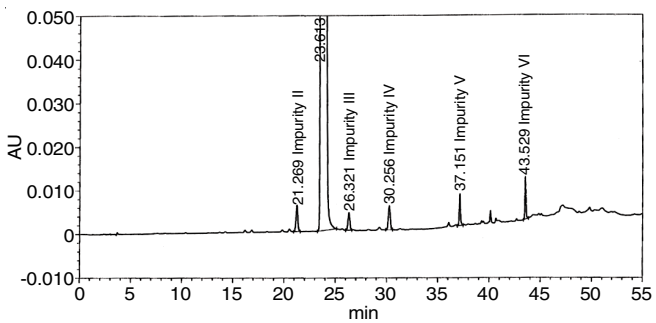

Fig. 2. Representative HPLC chromatogram of linagliptin drug substance – spiked with linagliptin regioisomer, reverse linagliptin, diquinazolinyl linagliptin, bromo butene linagliptin and dimethylamino linagliptin

Method validations: Proposed related substances method of linagliptin has been validated as per ICH guidelines [17] to prove its competence. The validation parameters studied were specificity, sensitivity, linearity, precision (repeatability and intermediate precision), accuracy and robustness.

System suitability: The linagliptin sample (enriched with lingliptin regioisomer and reverse lingliptin impurities) at a concentration level of 0.5 mg/mL in diluent was injected into the HPLC system and monitored for system suitability. Resolution between "lingliptin regioisomer, linagliptin" and "linagliptin, reverse linagliptin" is a minimum of 2. The column efficiency as determined from the linagliptin peak is not less than 8000 USP plate counts.

Specificity: The specificity and stability indicating nature of the proposed method for related substances were performed in the presence of forced degradation. The wet and dried forced degradation studies of linagliptin were subjected to acid, base, peroxide, thermal, photolytic and humidity conditions. Stress studies were performed at a test concentration level for the linagliptin drug substance. Conditions are tabulated in Table-1. Stressed samples were analyzed by using the PDA detector to evaluate the ability of the proposed method to separate all the related substances (process related) from the degradation products. Peak purities were calculated by using empower software and found that the purity angle was within the purity threshold limit in all stressed samples, which concluded the homogeneity of the analyte peak (Table-1). The proposed related substances were well separated from the obtained degradation products. Therefore, the proposed method was specific. Representative HPLC chromatograms for the forced degradation of linagliptin drug substance are shown in Fig. 3.

Limit of detection and limit of quantification: The sensitivity of an analytical method was demonstrated in terms of the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ concentrations were estimated for linagliptin and its related substances by using the slop of the linearity curve, ranging from 5 to 150% of the specification level. Predicted concentrations of LOD and LOQ solutions for linagliptin and its related substances were prepared and injected into HPLC as per the methodology. The % RSD values for the precision of linagliptin and its related substances were ranged from 1.1-21.9 and 0.7-4.0 for LOD and LOQ, respectively (Table-2). Thus, the proposed method had adequate sensitivity for the detection and quantification of related substances in linagliptin.

Linearity: Linearity was performed by injecting ten diluted solutions ranging from LOQ to 150% of the specification level. The correlation coefficients for linagliptin and its related substances from the linear graph were found to be in the range between 0.9990 and 0.9999. The results are shown in Table-3.

Accuracy: Accuracy was determined by the standard addition method, in which known quantities of related substances of linagliptin were spiked into the linagliptin sample

TABLE-1
SPECIFICITY

Degradation	Degradation condition	Area	Degradation (%)	Peak purity of linagliptin	
				Purity angle	Purity threshold
–	Undegraded sample	14598540	–	0.057	0.258
Acid	5 M HCl/85 °C/120 min	14174328	2.9	0.061	0.257
Base	5 M NaOH/85 °C/30 min	12774723	12.4	0.069	0.256
Peroxide	30% H_2O_2/RT/30 min	12220189	16.3	0.063	0.286
Thermal	105 °C/120 h	14280236	2.2	0.053	0.257
Photolytic	Fluorescent light: 1.2 million lux hours; UV light: 200 watt h/m^2	13912136	4.7	0.063	0.256
Humidity	90% RH/25 °C/120 h	14526072	0.5	0.06	0.257

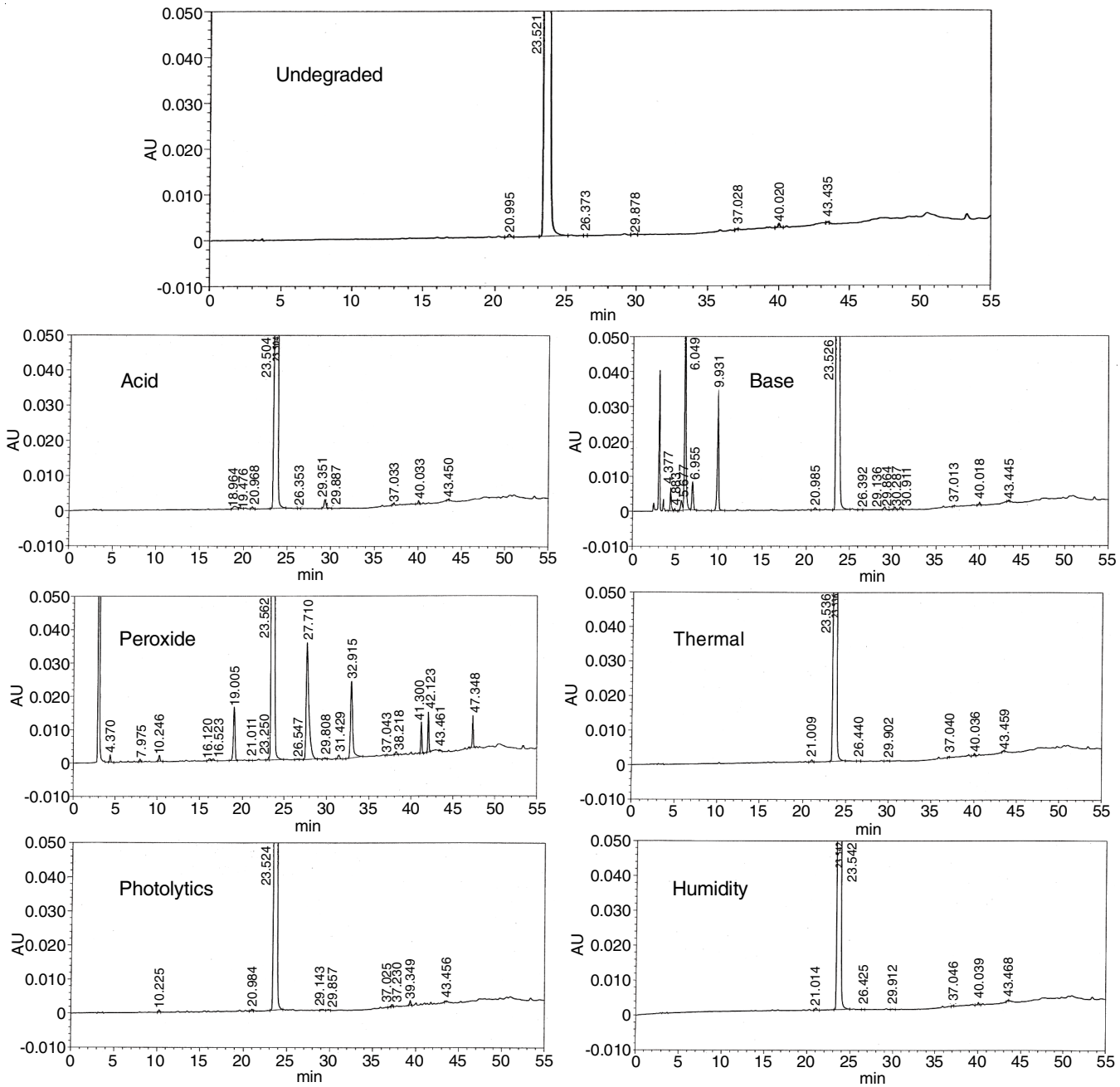

Fig. 3. Representative HPLC chromatograms of linagliptin drug substance-forced degradation experiments

TABLE-2
LOD & LOQ PRECISION

Name of the impurity	Average peak area		% RSD		Concentration (% w/w)	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Linagliptin regioisomer	1687	4952	2.0	0.8	0.005	0.014
Linagliptin	2918	5929	21.9	4.0	0.005	0.014
Reverse linagliptin	1587	4939	3.5	0.8	0.005	0.014
Diquinazolinyl linagliptin	1932	5847	2.4	1.5	0.004	0.013
Bromo butene linagliptin	1671	4931	1.1	0.7	0.005	0.015
Dimethylamino linagliptin	2897	7089	9.3	1.0	0.005	0.014

solution. Spiked solutions were prepared in triplicate at the LOQ level and 50-150 % w/w level. The related substances of linagliptin were determined from the spiked sample solutions and the percent recovery was calculated and it was found to lie in between 91.8 and 103.1%w/w. The statistical analysis showed that the method was accurate (Table-4).

TABLE-3
LINEARITY

Name of the impurity	Conc. range (µg/mL)	Slope	Intercept	Correlation coefficient	Residual sum of squares	Response factor
Linagliptin regioisomer	0.069-1.105	70571	159	0.9998	502	1.12
Linagliptin	0.071-1.133	79099	396	0.9998	650	1.00
Reverse linagliptin	0.071-1.126	69681	16	0.9998	461	1.14
Diquinazolinyl linagliptin	0.066-1.142	87458	185	0.9999	490	0.9
Bromobutene linagliptin	0.077-1.149	64017	83	0.9997	633	1.24
Dimethylamino linagliptin	0.069-1.127	88459	668	0.9990	1592	0.89

TABLE-4
ACCURACY

Accuracy parameter	Linagliptin regioisomer				Reverse linagliptin				Diquinazolinyl linagliptin			
	LOQ level	50% level	100% level	150% level	LOQ level	50% level	100% level	150% level	LOQ level	50% level	100% level	150% level
Amount added (% w/w)	0.0136	0.075	0.15	0.225	0.0135	0.076	0.153	0.229	0.0127	0.077	0.154	0.230
Amount found (% w/w)	0.0133	0.072	0.143	0.215	0.0126	0.073	0.146	0.221	0.012	0.076	0.151	0.226
% Recovery	97.8	95.6	95.6	95.6	93.3	95.6	95.6	96.5	95.0	98.7	98.1	98.0
% RSD	2.6	0.8	0.4	0.5	1.6	1.6	0.3	0.5	0.5	0.0	0.1	0.7
Overall % Recovery	96.2				95.3				97.5			

Accuracy parameter	Bromo butene linagliptin				Dimethylamino linagliptin			
	LOQ level	50% level	100% level	150% level	LOQ level	50% level	100% level	150% level
Amount added (% w/w)	0.015	0.075	0.149	0.223	0.0136	0.071	0.141	0.212
Amount found (% w/w)	0.0148	0.074	0.151	0.226	0.0129	0.067	0.132	0.197
% Recovery	98.5	99.1	101.3	101.3	94.9	94.4	93.2	92.9
% RSD	2.4	1.6	0.6	1.8	2.0	1.5	0.4	1
Overall % Recovery	100.1				93.9			

Precision: The system precision has been demonstrated by injecting six replicate injections of standard solution into the HPLC system and the %RSD of area response for six replicate measurements was found to be less than 5. Method precision has been evaluated by means of repeatability and intermediate precision. To determine the repeatability and intermediate precision of the method, replicated (n = 6) injections of the spiked sample at specification level of related substances of linagliptin were carried out and precision was expressed as % RSD. The results are presented in Table-5.

Robustness: Robustness of the method indicates the ability of the method to remain unchanged even after variations in critical method parameters. The effect of variation in flow, percentage organic in gradient composition, pH of the buffer, column oven temperature and detection wavelength on system suitability criteria was studied. The resolutions between "lingliptin regioisomer, linagliptin" and "linagliptin, reverse linagliptin" were found to be more than 4.8 in each variation, which indicates the robustness of the method. The statistical analysis of robustness data is illustrated in Table-6.

TABLE-5
METHOD PRECISION (MP) AND INTERMEDIATE PRECISION (IP)

Sample ID	Linagliptin regioisomer		Reverse linagliptin		Diquinazolinyl linagliptin		Bromo butene linagliptin		Dimethylamino linagliptin	
	MP	IP	MP	IP	MP	IP	MP	IP	MP	IP
Sample-1	0.191	0.202	0.137	0.145	0.158	0.163	0.172	0.163	0.163	0.146
Sample-2	0.195	0.205	0.140	0.149	0.161	0.164	0.174	0.166	0.165	0.150
Sample-3	0.185	0.206	0.120	0.146	0.152	0.165	0.167	0.166	0.157	0.151
Sample-4	0.190	0.204	0.136	0.146	0.158	0.161	0.170	0.164	0.162	0.151
Sample-5	0.191	0.204	0.137	0.146	0.159	0.164	0.172	0.163	0.163	0.145
Sample-6	0.196	0.204	0.140	0.147	0.163	0.162	0.176	0.161	0.167	0.150
Mean	0.191	0.204	0.137	0.147	0.159	0.163	0.172	0.164	0.163	0.149
S.D.	0.004	0.001	0.003	0.001	0.004	0.001	0.003	0.002	0.003	0.003
% RSD	2.1	0.5	2.2	0.7	2.5	0.6	1.7	1.2	1.8	2.0
95% confidence interval (±)	0.004	0.003	0.003	0.001	0.004	0.001	0.003	0.002	0.003	0.003
Overall statistical data:										
Mean	0.198		0.142		0.161		0.168		0.156	
S.D.	0.007		0.005		0.004		0.005		0.008	
% RSD	3.5		3.5		2.5		3.0		5.1	
95% confidence interval (±)	0.004		0.003		0.003		0.003		0.003	

TABLE-6
ROBUSTNESS

Condition	Variation	USP plate count	USP resoution*	USP resoution#
Existing	–	24624	5.1	5.6
Flow rate	-10%	26148	5.0	5.7
	10%	23371	5.1	5.6
% of Organic in gradient composition	-2% absolute	30703	5.2	5.7
	+2% absolute	19203	4.8	5.5
pH of Buffer	-0.2 Units	25150	5.1	5.7
	+0.2 Units	24943	5.1	5.7
Column oven temperature	-5 °C	25086	4.9	5.7
	+5 °C	23273	5.1	5.5
Wavelength	-3 nm	22864	4.9	5.6
	+3 nm	22774	4.9	5.6

*USP resolution between Linagliptin regioisomer and Linagliptin; #USP resolution between Linagliptin and Reverse linagliptin

Solution stability: Stability of linagliptin solutions at test concentration and standard level were studied by keeping the solution at room temperature on a laboratory bench top for 24 h. Related substances were determined at 65 min interval during the study period. The standard solution was stable for at least 24 h at room temperature and the sample solution was not stable at room temperature and refrigerator condition.

Conclusion

A reverse phase high performance liquid chromatography method has been developed, optimized and validated for the determination of related substances of a DPP-4 inhibitor drug linagliptin. The results from the validation experiments proved that the optimized related substances method was specific (stability indicating), sensitive, linear, precise, accurate, robust and rugged for the determination of related substances in linagliptin drug substance and can be used in the routine.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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