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Article

Stability-Indicating RP-LC Method for the Determination of Vildagliptin and Mass Spectrometry Detection for a Main Degradation Product

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A simple, precise and stability-indicating reversed-phase liquid chromatography method was developed and validated for the determination of vildagliptin (VLG) in pharmaceutical dosage form. The chromatographic separation was obtained within 6 min and was linear in the range of 20–80 μg/mL ($r^2 = 0.9999$). Limit of detection and limit of quantitation were 0.63 and 2.82 μg/mL, respectively. The method was validated in accordance with International Conference on Harmonization acceptance criteria for specificity, linearity, precision, accuracy, robustness and system suitability. Stress studies were carried out and no interference of the degradation products was observed. The excipients did not interfere in the determination of VLG. Furthermore, the main degradation product obtained from the stress studies (thermal, oxidative and alkaline hydrolysis) was evaluated for mass spectrometry and its molecular structure was predicted. The proposed method was successfully applied for the quantitative analysis of VLG in tablet dosage form, which will help to improve quality control and contribute to stability studies of pharmaceutical tablets containing this drug.

Introduction

Dipeptidyl peptidase 4 (DPP4) inhibitors represent a new class of antidiabetic agents that improve glycemic control by preventing glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) degradation. These intestinal peptides, also known as incretins, are postprandially secreted and lead to a rise in insulin secretion (1).

Vildagliptin (VLG), (S)-1-[*N*-(3-hydroxy-1-adamantyl)glycyl] pyrrolidine-2-carbonitrile, is a potent and selective DPP4 inhibitor that improves glycaemic control in patients with type 2 diabetes mellitus by increasing α- and β-cell responsiveness to glucose (2). The chemical structure of the drug is shown in Figure 1.

The pharmaceutical product is commercially available, but currently no methods are published for the quantitative analysis of VLG as an active pharmaceutical ingredient or finished product.

Therefore, the purpose of this research work was to develop and validate a simple, fast, accurate and stability-indicating reversed-phase liquid chromatography (RP-LC) method for the determination of VLG in solid pharmaceutical dosage form.

Experimental

Reagents and chemicals

The reference standard (purity of 99.5%) was purchased from Sequoia Research Products and the commercial tablets of Galvus (Novartis Biociências) containing 50 mg of VLG were obtained from commercial sources within their shelf-life period. LC grade acetonitrile was obtained from Tedia (Fairfield, OH). All chemicals used were of pharmaceutical or special analytical grade. Purified water was obtained by a Millipore Direct-Q 3UV with pump (Molsheim).

Apparatus and analytical conditions

Liquid chromatography

The LC method was carried out on an Agilent liquid chromatograph (Santa Clara, CA) equipped with a model Q1311A quaternary pump, ALS-G1329A auto sampler, TCC-G1316A column oven and G1315B photodiode-array detector, and ChemStation manager system software was used to control the equipment and to calculate data and responses from the LC system. The experiments were performed on an XBridge analytical column C8 (150 × 4.6 mm i.d., 5 μm particle size) (Waters). The LC system was operated isocratically, at room temperature, using a mobile phase consisting of acetonitrile and a solution of triethylamine 0.3% adjusted to pH 7.0 with phosphoric acid (15:85; *v*/*v*) run at a flow-rate of 1.0 mL/min, and using photodiode array (PDA) detection at 207 nm. The injection volume was 20 μL of the solutions containing 50 μg/mL for working standard and sample solutions.

Mass spectrometry

Mass spectrometric (MS) analysis was performed using a Quattro Micro (triple-quadrupole) instrument from Micromass (Manchester, UK) working with an electrospray ionization interface operating in a positive mode (ESI+), set up in scan mode. The mass spectrometer conditions were optimized with the direct injection (Baby Bee syringe pump, Bioanalytical Sciences, West Lafayette, IN) of the VLG reference solution and its solutions submitted to a thermal degradation process, oxidation and alkaline hydrolysis into the system at a concentration of 2 μg/mL. The best response was obtained with an ESI capillary potential of 3.2 kV, cone voltage of 30 V, RF lens voltage of 0.3 V, source temperature of 120°C and ESI probe temperature of 350°C. Before analysis, the samples were diluted in methanol–water (50:50, *v*/*v*), and introduced into the mass spectrometer by direct infusion at 10 μL/min. The data acquisition and system control were obtained using MassLynx version 3.5 software from Micromass. The MS data were acquired in the *m*/*z* range between 100 and 500 amu.

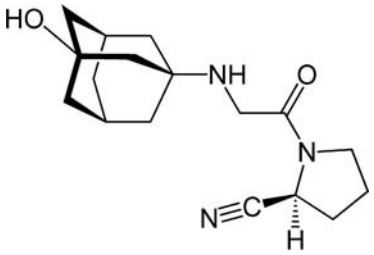

Figure 1. Chemical structure of vildagliptin (VLG).

Solutions

Preparation of reference substance solutions

Stock solutions were prepared by accurately weighing 50 mg of VLG reference substance, transferring the reference substance to individual 100-mL volumetric flasks and diluting to volume with water, obtaining a concentration of 0.5 mg/mL. The stock solutions were stored at 2–8°C, protected from light and diluted daily to an appropriate concentration in mobile phase.

Preparation of sample solutions

Tablets containing 50 mg of VLG were accurately weighed and crushed to fine powder. An appropriate amount was transferred into an individual 100-mL volumetric flask, diluted to volume with water, sonicated for 10 min and filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA), obtaining a concentration of 0.5 mg/mL of VLG. These stock solutions were stored at 2–8°C and protected from light. Working sample solutions were prepared daily by diluting the stock solutions to the appropriate concentration in mobile phase.

Method Validation

The method was validated using samples of pharmaceutical formulation with the label claim of 50 mg of VLG by determining the following parameters: specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness following the International Conference on Harmonization (ICH) guidelines (3, 4). Moreover, stability-indicating capability was demonstrated by performing stress studies, also called forced degradation (5).

Specificity

The sample solutions (0.5 mg/mL of VLG) were subjected to accelerated degradation to evaluate interference in the quantification of the compound and to show the stability-indicating property and specificity of the proposed method. Interference of the pharmaceutical formulation excipients was determined by the injection of a sample containing only placebo (in-house mixture of all the tablet excipients) and a sample containing placebo with VLG added at a concentration of 50 μg/mL. The PDA detector was used and the stability-indicating capability of the method was established for the peak purity test by determining the peak purity of VLG in the degraded samples. The analyzed stress conditions were:

(i) Acid hydrolysis: sample solution was prepared and maintained in 1.0M hydrochloric acid for 24 h and stored at room temperature. After that, 5.0 mL of this solution were removed and neutralized with 1.0M sodium hydroxide and diluted to a concentration of 50.0 μg/mL in mobile phase.

(ii) Basic hydrolysis: sample solution was prepared and maintained in 0.1M sodium hydroxide for 2 h and stored at room temperature. After that, 5.0 mL of this solution were removed and neutralized with 0.1M hydrochloric acid and diluted to a concentration of 50.0 μg/mL in mobile phase.

(iii) Oxidative degradation: sample solution was prepared in 0.3% H_2O_2 solution and stored at room temperature for 2 h. After that, 5.0 mL of this solution were removed and diluted to a concentration of 50.0 μg/mL in mobile phase.

(iv) Thermal degradation: sample solution was exposed at 60°C for 240 h. After that, 5.0 mL of this solution were removed and diluted to a concentration of 50.0 μg/mL in mobile phase.

(v) Photodegradation: sample solutions were induced by exposing to 200 Wh/m^3 of near UV light emitting UV-A radiation (352 nm) and UV-C radiation (254 nm) for 24 h. Control samples were protected from light with aluminum foil and, similarly, were exposed to radiation. The study was performed exposing the solutions in quartz cells in the photodegradation chamber. After that, these solutions were diluted to a concentration of 50.0 μg/mL in mobile phase.

Linearity

Linearity was determined by independently constructing three analytical curves, each with seven reference substance concentrations in the 20–80 μg/mL range of VLG, prepared in mobile phase. The peak areas of the chromatograms were plotted against the respective concentration of VLG to obtain the analytical curve. The results were subjected to regression analysis by the least squares method to calculate the calibration equation and determination coefficient, and by analysis of variance for compliance of the linear model.

Precision and accuracy

The method precision was determined by repeatability and intermediate precision. Repeatability was verified by six independent sample preparations of the same concentration of VLG, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-days) and also by other analysts performing the analysis in the same laboratory (between-analysts). The accuracy was determined by the recovery of known amounts of VLG reference substance added to the samples in the beginning of the preparation process. The added levels were 25, 50 and 75% of the nominal drug concentration. The results were expressed as the percentage of VLG reference substance recovered from the sample.

Limits of detection and quantitation

The LOD and LOQ were calculated and obtained experimentally based on the signal-to-noise approach. The background noise was obtained after injection of the blank and observed over a distance equal to 20 times the width at half height of the peak in a chromatogram obtained by the injection of 50 μg/mL of

Table I
Factors and Levels Investigated during Robustness Testing

Factor	Nominal	Levels investigated (−1; +1)
pH of aqueous phase	7.0	6.8; 7.2
% of triethylamine	0.3	0.28; 0.32
% of acetonitrile	15	14; 16
Temperature (°C)	25	23; 27
Flow (mL/min)	1.0	0.8; 1.2
Column manufacturer	Waters	Ace; Waters

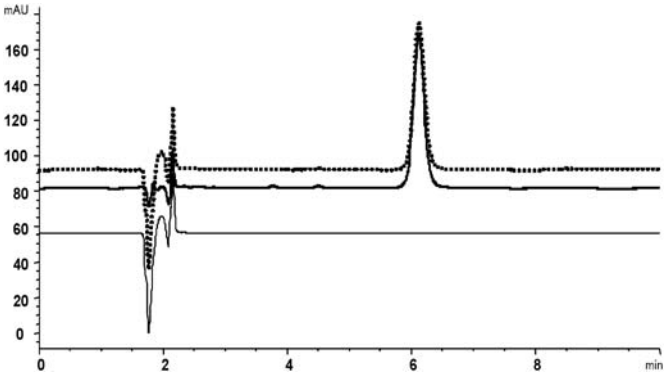

Figure 2. Overlapping representative RP-LC chromatograms of vildagliptin (VLG) sample solution (A), reference substance solution (B); and placebo solution (C). Chromatographic conditions: XBridge C8 column (150 × 4.6 mm, 5 μm), 25°C; mobile phase: triethylamine (0.3%; pH 7.0)–acetonitrile (85:15; *v/v*); flow rate: 1.0 mL/min; detection: 207 nm.

the reference substance. The applied signal-to-noise ratio was 10:1 for LOQ and 3:1 for LOD (6).

Robustness

VLG standard and sample were analyzed under identical experimental conditions. Robustness was investigated with the factors summarized in Table I.

Solution stability

To assess the stability of sample solutions of VLG, the samples were tested. They were maintained at 2–8°C for 72 h and also placed into the autosampler at room temperature for 24 h. The stability of these solutions (compared with freshly prepared solutions) and of the mobile phase was studied and any change in the chromatographic pattern was observed.

System suitability

The system suitability test was also carried out to evaluate the reproducibility of the system for the analysis to be performed. Parameters measured in this study were peak area, retention time, theoretical plates, retention factor and peak asymmetry.

Results and Discussion

Selection and optimization of the chromatographic conditions

The effect of the column (C8 and C18) and mobile phase composition on the retention time of VLG and its

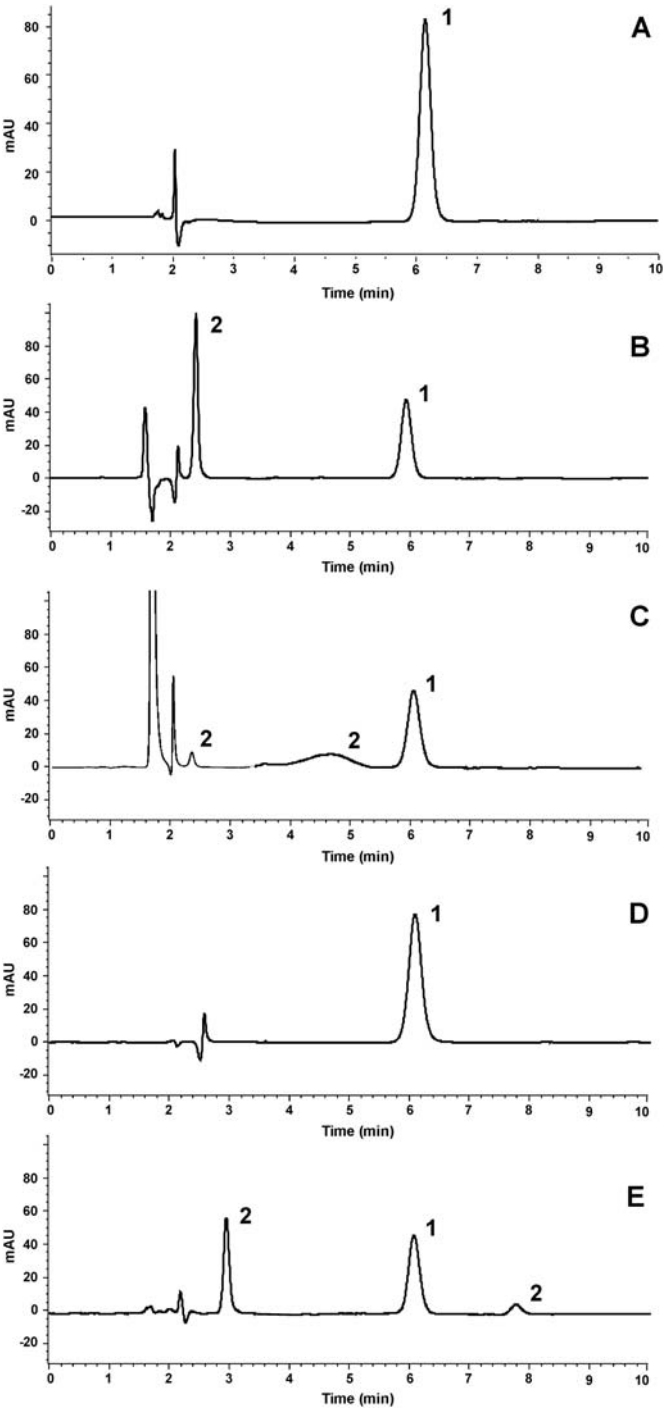

Figure 3. RP-LC chromatograms of vildagliptin drug product (VLG; 50 μg/mL) obtained: acidic hydrolysis (A); basic hydrolysis (B); oxidative degradation (C); photodegradation (D); thermal degradation (E). Peak 1: VLG, 2: degraded form, 3: hydrogen peroxide. Chromatographic conditions: XBridge C8 column (150 × 4.6 mm, 5 μm), 25°C; mobile phase: triethylamine (0.3%; pH 7.0)–acetonitrile (85:15; *v/v*); flow rate: 1.0 mL/min; detection: 207 nm.

chromatographic parameters was initially investigated. The best chromatographic conditions were chosen after the column test where C8, compared to C18, presented appropriate chromatographic parameters. Different mobile phases were tested in distinct proportions of organic solvent: water and acetonitrile

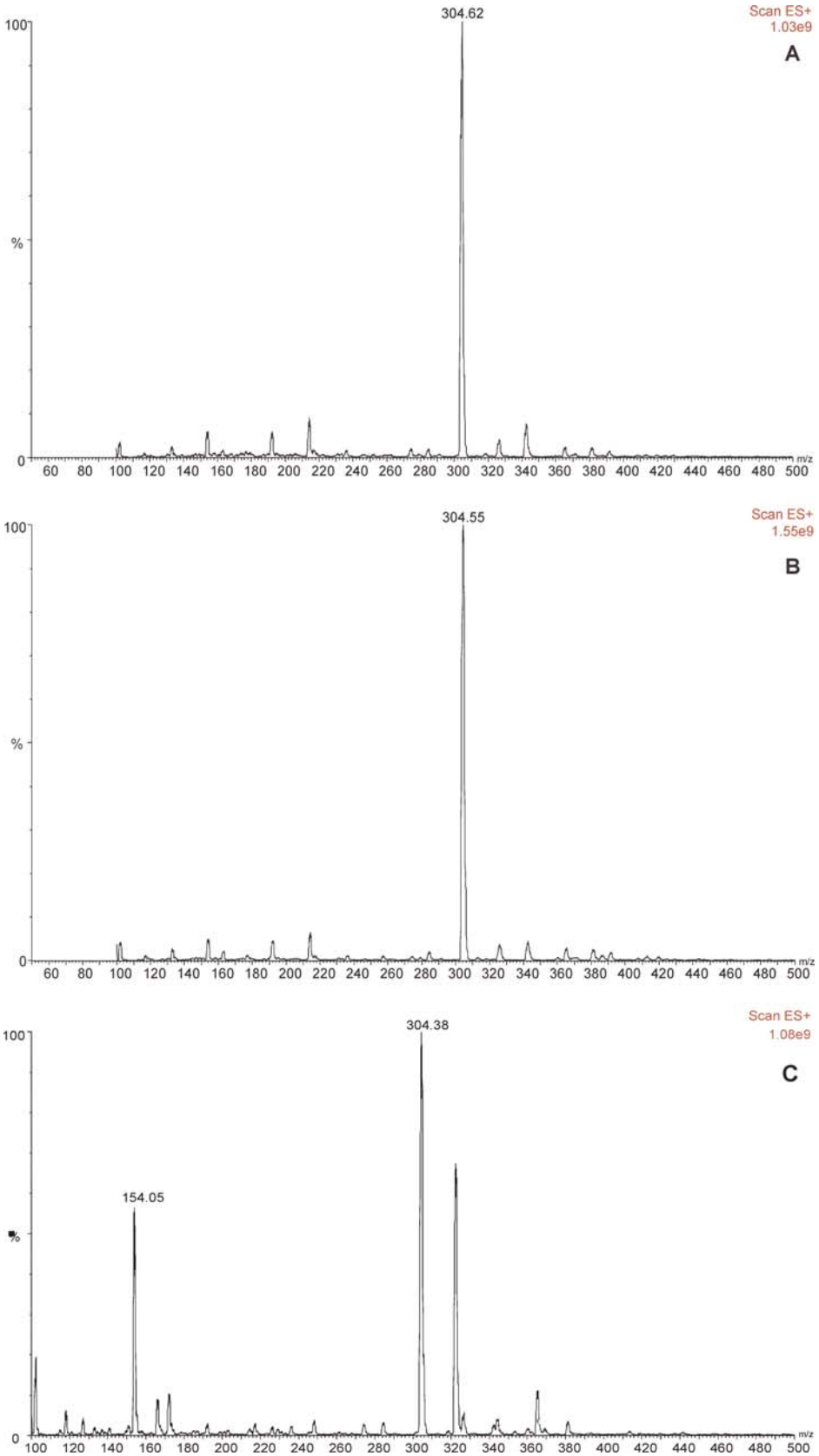

Figure 4. Mass spectra obtained for vildagliptin: reference substance (A); sample (B); thermal degradation (C); basic hydrolysis (D); oxidative degradation (E).

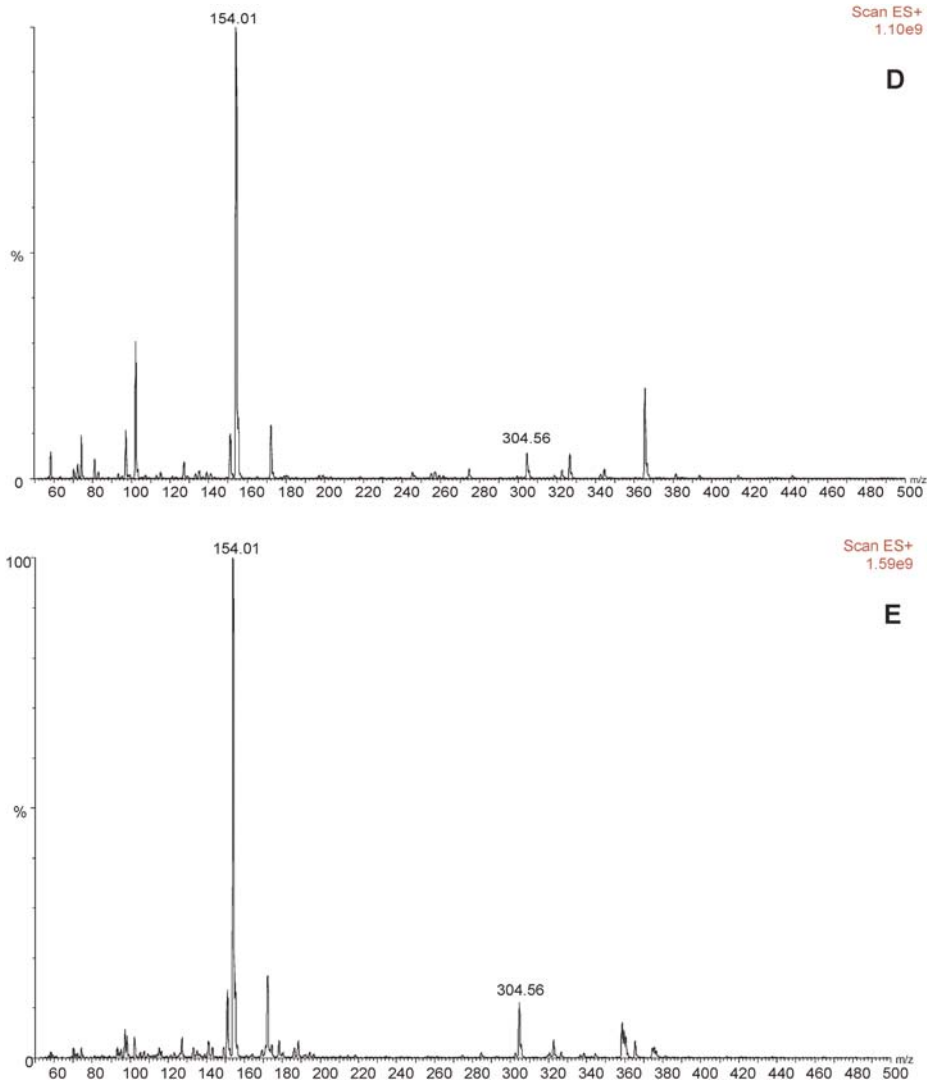

Figure 4. Continued

(80:20, 83:17, 85:15, 87:13; *v*/*v*), water and methanol (85:15 *v*/*v*) and water, acetonitrile and methanol (50:20:30, 60:20:20, 80:15:5; *v*/*v*), adjusted with triethylamine, at different pH values of the aqueous phases (5.0, 6.0, 7.0, 8.0 and 9.0). The adequacy of the mobile phase was decided on the basis of assay selectivity and sensitivity, stability studies and the separation between the degradation products formed during stress studies. The system suitability test was appropriate using triethylamine solution (0.3%; pH 7.0) in combination with acetonitrile (85:15; *v*/*v*), at room temperature ($23 \pm 1^{\circ}C$), which resulted in a retention time of 6.2 min, and a simple mobile phase (without salt buffer addition), as shown in the typical chromatogram in Figure 2. VLG was determined at 207 nm and no interference was observed, therefore, this wavelength was utilized. The optimized conditions of the RP-LC method were validated for the analysis of VLG in pharmaceuticals formulations, due to capability and application for quality control.

Method validation

Specificity and forced degradation studies

Forced degradations were performed to show the stability-indicating properties of the analytical method, particularly when there is no information available about the potential degradation products. For VLG, the oxidative and basic conditions resulted in a significant decrease of the area with additional peaks. Under the basic condition, a significant decrease of the peak area was observed within 2 h, with one additional peak detected at 2.4 min (peak 2, Figure 3B). Under the acid condition, no significant decrease in the area of the VLG was exhibited. The acid and photolytic conditions resulted in non-significant reduction of the peak area with no additional peak detected by UV (Figures 3A and 3D). Under the oxidative condition, a significant decrease of the area of VLG was detected and two small degradation product peaks were seen at approximately 2.4 and 4.1 min (peak 2, Figure 3C). Under

the thermal condition, it was observed that the area decreased within 240 h, with two additional peaks detected at 2.9 and 7.8 min (peak 2, Figure 3E). No interference from formulation excipients was found, showing that the peaks were free from any coeluting peak, thus demonstrating that the proposed method is specific for the analysis of VLG. The stressed samples were, respectively, analyzed and compared to the VLG reference solution spectrum, and with the help of PDA the peak purity of VLG was verified in the degraded samples. Moreover, subsequent studies used mass spectrometry to analyze the reference substance and the main degraded samples. The chromatograms of forced degradation studies are shown in Figure 3.

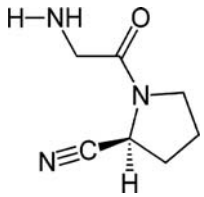

Figure 5. Possible major degradation product formed under all degradation conditions.

Table II
Inter-Day and Between-Analyst Precision Data of RP-LC for Vildagliptin in Pharmaceutical Formulation Samples

Inter-day		Between-analyst	
Day	Assay (%)	Analysts	Assay (%)
	97.36		97.66
	96.79		97.42
1	96.88	A	97.44
	97.26		97.86
	97.32		98.08
	99.23		98.60
	97.43		97.43
	97.12		97.12
2	96.90	B	96.90
	97.97		97.97
	97.97		97.97
	96.82		96.82
	97.96	Mean†	97.61
	98.06	RSD (%)	0.59
3	97.99		
	98.45		
	98.85		
	97.96		
Mean*	97.68		
RSD (%)	0.71		

*Mean of 18 replicates.
†Mean of 12 replicates.

Mass spectrometry

Through analysis of the mass spectrum of solutions obtained in forced degradation studies; specifically, thermal, oxidative and alkaline degradations, it was possible to predict the likely molecular structure of the major degradation product formed under these conditions. The presence of the main degradation product can be seen in all conditions at the peak of 154 (m/z). The spectra are shown in Figure 4 and the possible main degradation product formed is shown in Figure 5. Furthermore, analysis by LC–MS-MS and by nuclear magnetic resonance will be needed to confirm the chemical structure.

Linearity

The analytical curves constructed for VLG were found to be linear in the 20–80 μg/mL range. The value of the determination coefficient calculated ($R^2 = 0.9999$, $y = 2126352.79x + 12287.36$, where x is concentration and y is the peak absolute area) indicated the linearity of the analytical curve for the method. The variance analysis ($P = 0.05$) was applied to verify the linearity of the method and the results showed that the regression equation was linear ($F_{calculated} = 77977.56 > F_{critical} = 4.40$) with no deviation from linearity ($F_{calculated} = 1.69 < F_{critical} = 4.53$).

Precision

The precision evaluated as the repeatability of the method was studied by calculating the relative standard deviation (RSD) for six determinations of 50 μg/mL, performed on the same day and under the same experimental conditions. The RSD value obtained was 0.61. The inter-day precision was assessed by analyzing six samples on three different days; the mean values obtained were 97.68% (RSD = 0.71). Between-analyst precision was determined by two analysts analyzing six samples; the values were found to be 97.61% (RSD = 0.59). These results are summarized in Table II.

Accuracy

Accuracy was evaluated by determining the analyte in solutions prepared according to the standard addition method and expressed in terms of percentage recoveries of VLG from the real samples. The results are shown in Table III. The mean recovery data were 97.71% (RSD = 0.31), demonstrating that the method is accurate within the desired range.

Limits of detection and quantitation

The LOD and LOQ were obtained by using the mean of the slope and the standard deviation of the intercept of the independent curves, determined by a linear regression line. The calculated LOD was 0.46 μg/mL and the LOQ was 1.52 μg/mL.

Table III
Accuracy of RP-LC in Pharmaceutical Formulation Samples

Drug	Added level (μg/mL)	Nominal concentration (μg/mL)	Mean concentration found* (μg/mL)	Accuracy (%)	RSD (%)
VLG (40 μg/mL)	10.0 (25%)	50.0	48.07	97.90	0.31
	20.0 (50%)	60.0	57.86	97.88	
	30.0 (75%)	70.0	67.49	97.35	

*Mean of three replicates.

Table IV
Responses* Obtained Compared to the Standard Solutions after Changes in Factors Investigated by the Robustness Test

Modified factors	Drug obtained (%)	RSD (%)
Aqueous phase (pH 6.8)	97.49	0.24
Aqueous phase (pH 7.2)	96.74	0.79
Mobile phase proportion (84:16)	97.47	0.25
Mobile phase proportion (86:14)	97.89	0.05
Temperature (23°C)	97.89	0.05
Temperature (27°C)	97.91	0.07
Flow (0.8 mL/min)	97.79	0.02
Flow (1.2 mL/min)	98.31	0.35
Column manufacturer (Ace)	98.69	0.63
Column manufacturer (Waters)	97.82	—

*Percentage of VLG in the commercial tablets relative to the concentration claimed in its label.

The experimentally determined LOD and LOQ were 0.63 and 2.82 μg/mL, respectively.

Robustness

The responses (percentage of VLG in the commercial tablets relative to the concentration claimed on its label) obtained as compared to the standard solutions are summarized in Table IV. No significant changes were found in the chromatographic pattern when the modifications were made under experimental conditions, thus showing the method to be robust. The stability of the sample solutions was studied and the data obtained showed the stability during 24 h in the autosampler and during 72 h when maintained at 2–8°C.

System suitability

Analysis of the VLG standard evaluated daily presented approximately these results: 6,345 of theoretical plates and 0.99 of peak asymmetry. The obtained retention factor value was 3.60. The experimental results show that the parameters tested were within the acceptable range, indicating that the system is suitable for the intended analysis (7).

Conclusion

Results of the validation studies show that the stability-indicating RP-LC method is specific, accurate and robust and possesses significant linearity and precision, without any interference from the excipients and degradation products. The proposed method was successfully applied for the quantitative analysis of VLG in tablets, and may thus be used for routine analysis, quality control and studies of the stability of pharmaceutical tablets containing this drug.

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