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Capillary zone electrophoresis for determination of vildagliptin (a DPP-4 inhibitor) in pharmaceutical formulation and comparative study with HPLC

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A stability-indicating capillary zone electrophoresis (CZE) method was validated for the determination of vildagliptin (VLG) in pharmaceutical dosage forms using ranitidine hydrochloride (RH) as internal standard. The CZE method was carried out in a fused silica capillary (64.5 cm total length and 56.0 cm effective length, 50 μm i.d.) by applying a potential of 25 kV (positive polarity), hydrodynamic injection by 50 mbar for 5 s and the temperature of the capillary cartridge was 25 °C. The selected background electrolyte (BGE) consisted of 25 mM potassium phosphate (pH 8.0) with UV/PDA detection at 207 nm. The electrophoretic separation was obtained within 6 min and was linear in the range of 50–200 μg/mL (r = 0.9994). The specificity and the stability-indicating capability were demonstrated through degradation studies, which also showed that there was no interference of the formulation excipients. The method was validated in accordance to ICH guidelines acceptance criteria for specificity, linearity, precision, accuracy, robustness and system suitability. The proposed method was compared with HPLC method previously validated for this drug, and statistical analysis showed no significant difference between the methods.

1. Introduction

The interaction between environmental and genetic factors results in the development of insulin resistance and β-cell dysfunction which characterizes the dysfunction called Type 2 Diabetes mellitus (Facchini et al. 2001; Stumvoll et al. 2005; Tahrani et al. 2010). In order to improve glycemic control and slowing disease progression, pharmacological and non-pharmacological alternatives have been developed. In relation to pharmacological intervention the treatment with DPP-4 inhibitors have been considered (Kahn et al. 2006; Del et al. 2007).

Vildagliptin (VLG), (2*S*)-1-[*N*-(3-hydroxy-1-adamantyl) glycyl] pyrrolidine-2 (Fig. 1) is an active oral DPP-4 inhibitor, one of the most studied of the new class of incretin enhancers. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are intestinal peptides (called also as incretins) produced in the gut and integrally involved in regulation of α- and β-cell function. They are rapidly degraded during circulation by the enzyme dipeptidyl peptidase-4 (DPP-4) (Jost et al. 2009; Mathieu 2009). VLG provides glycemic control by inhibiting the rapid degradation of the incretins enabling higher levels of active hormone to be maintained and available for glucose-mediated modulation of islet cell function (Bolli et al. 2009).

Capillary electrophoresis (CE) is currently a powerful and established method for pharmaceutical analysis and is recommended in several pharmacopeias as The British Pharmacopeia and The United States Pharmacopeia (USP 34 2011; BP 2012). Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are the two most widely used separation modes of this technique, used for analyses of various substances,

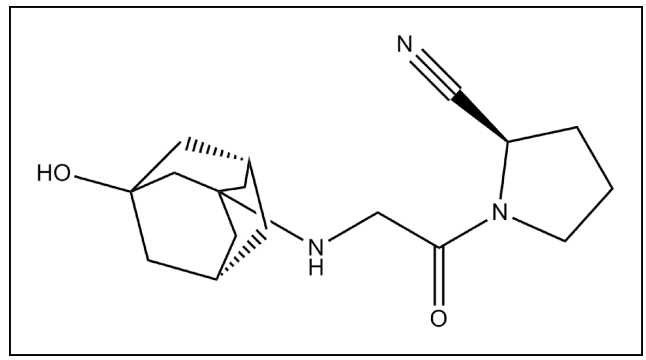

Fig. 1: Chemical structure of vildagliptin.

including active pharmaceutical ingredients (either in formulations or biological fluids), drug counter ions and impurities (Terabe 2008; Petersen et al. 2003).

CE is important for quality control of pharmaceutical products for both qualitative and quantitative analyses and has, nowadays, equal importance to HPLC in drug analysis. Quantitative analysis is mainly determined by comparison of migration time of the compounds of interest with those of the standards. Quantitative analysis is calculated from the peak area based on a calibration curve of a standard. CE separation depends on different migration of solutes in an electric field and electrophoresis is performed in narrow-bore capillaries filled with background electrolyte (BGE) (Suntornsuk 2010; Altria and Elder 2004).

As at the moment there is no published CE method for VLG, the aim of the present study was to develop and validate a stability-indicating CZE method for the determination of the drug in

solid pharmaceutical dosage forms, and of compose it with a previously validated RP-LC method.

2. Investigations, results and discussion

2.1. Selection and optimization of the electrophoretic conditions

Electrophoretic conditions were selected testing some electrolyte solutions containing sodium and potassium phosphate, sodium acetate, sodium tetraborate and TRIS (in different molarities) by CZE in free solution. By this method it was possible to identify the vildagliptin peak at different concentrations in the range from 50 to 100 mM. The pH effect was also studied considering drug characteristics such as pK_a and the best results were obtained using potassium phosphate solution 25 mM at pH 8.0. The effect of the voltage (applied from cathode to anode) was investigated from 10 to 30 kV, as well as the capillary temperature ranging from 15 to 30 °C. The best results, considering the system adequability parameters (analysis time, asymmetry, theoretical plates and resolution), were achieved with 25 kV and 25 °C. The sample solutions were hydrodynamically injected at 50 mbar changing the injection time between 3 to 8 s, showing increased peak width and shape deformed after 5 s, thus injection time selected was 5 s. To compensate injection errors and minor fluctuations of the migration time, several substances were investigated as internal standard, including nimesulide, salicylic acid, butenafine, hydrochlorothiazide, atenolol and ranitidine hydrochloride which was chosen to be an adequate candidate considering its stability (no degradation face to forced degradation studies which the drug studied was submitted) and peak resolution. In order to improve peak intensity, the separation using MEKC method was investigated. The obtained results by MEKC were unsatisfactory since the vildagliptin peak did not appear in any of the studied situations (SDS and electrolyte solutions at concentrations from 5 to 100 mM and applied voltage from 10 to 25 kV).

2.2. Method validation specificity and forced degradation studies

Electrophoretic runs of a placebo solution and forced degradation studies were performed in order to provide the method stability indication. In order to evaluate the interference from formulation excipients, it was performed the comparison between the electropherograms obtained for solutions of vildagliptin reference standard and for simulated sample of excipients. There was interference of product components in the quantitative determination, as shown in Fig. 2A . The forced degradation studies in acid condition resulted in non-significant decrease of the area without any detectable eluting degradation product (Fig. 2B). However, under basic conditions the vildagliptin content exhibited a significant decrease of the area with additional resolved peak at 4.52 min (Fig. 2C).

The oxidative condition also exhibited decrease of the area with three additional peaks detected at 3.34, 4.18 and 4.42 min, respectively (Fig. 2D). In thermal condition there was decreasing of the peak area with presence of a minimum peak at 4.42 min (Fig. 2E). Finally, under photolytic conditions there was no vildagliptin degradation in the analyzed period (Fig. 2F). Specificity of the method towards the drug was established through determination of peak purity of the analyte and the IS in the working reference substance solution, obtained by overlay of the spectra captured using a PDA detector.

Linearity was verified in the concentration range of 50.0–200.0 μg/mL by the construction of three independent analytical curves each one with seven crescent points to vildagliptin concentration and internal standard in the same concentration (100 μg/mL). The linearity was evaluated by the least square regression analysis where the slope and intercept of the calibration curve were 0.0112 and 0.0114, respectively. The value of the determination coefficient calculated (r = 0.9994) have shown the method linearity. The analysis of variance (p = 0.05) was applied and the results demonstrated the regression equation linearity ($F_{calculated} = 124.97 > F_{critical} = 4.35$) with no deviation from linearity ($F_{calculated} = 1.05 < F_{critical} = 2.12$).

The LOD and LOQ were calculated by using the slope and the standard deviation of the intercept from the independent curves. The values obtained were 3.24 μg/mL and 9.82 μg/mL, for LOD and LOQ, respectively.

The precision of the method was determined by repeatability and intermediate precision. Both were studied by calculating the relative standard deviation (RSD) of the peak area ratio for vildagliptin determinations (sample solution) in the concentration of 100 μg/mL spiked with IS. The results obtained are summarized in Table 1.

Table 1: Precision of CZE method obtained for vildagliptin in tablet samples

	Intra-day precision (%)	RSD (%)
Day 1	97.85	1.03
Day 2	98.86	0.91
Day 3	99.26	0.82
	Inter-day precision (%)	RSD (%)
	98.66	0.74

The accuracy was evaluated by the determination of known VLG standard amounts added to the sample tablets solution and expressed in terms of percentage recoveries of VLG standard. For each level of concentration three determinations were performed. The results are shown in Table 2. The method was considered accurate, within the desired range, since the mean recovery data were 98.96% (RSD = 0.50) and bias lower than 1.36%.

In the robustness experiment, performed by Plackett–Burman design, some of the most important parameters such as electrolyte solution pH (7.9 and 8.1) and electrolyte concentration (24 and 26 mM), capillary temperature (24 and 26 °C) and voltage applied (24 and 26 kV) were evaluated. The responses (percentage of VLG in the commercial tablets relative to its label claimed concentration) obtained in relation to the standard solutions, changing all the parameters in the same time, are summarized in Table 3.

The effects for each parameter were calculated, by *t*-test, and none of the factors studied were significant, because the values calculated were smaller than the critical value of *t* (α= 0.05), (Fig. 3).

The electropherogram pattern compared to the optimized conditions, indicating that the proposed method is robust under the experimental conditions tested. The stability of the sample solutions was studied and the data obtained showed the stability during 24 h at room temperature (25 ± 1 °C) and during 48 h when maintained under refrigeration (2 ± 8 °C) with non-significant change (≤ 2%) relative to freshly prepared samples.

A system suitability test was carried out each day, to evaluate the reproducibility of the system using a reference substance solution containing 100 μg/mL of VLG. The RSD values calculated for migration time, peak area, peak symmetry and theoretical plates were within the acceptable range (RSD ≤ 2.0%) indicating the suitability of the proposed method for the intended analysis.

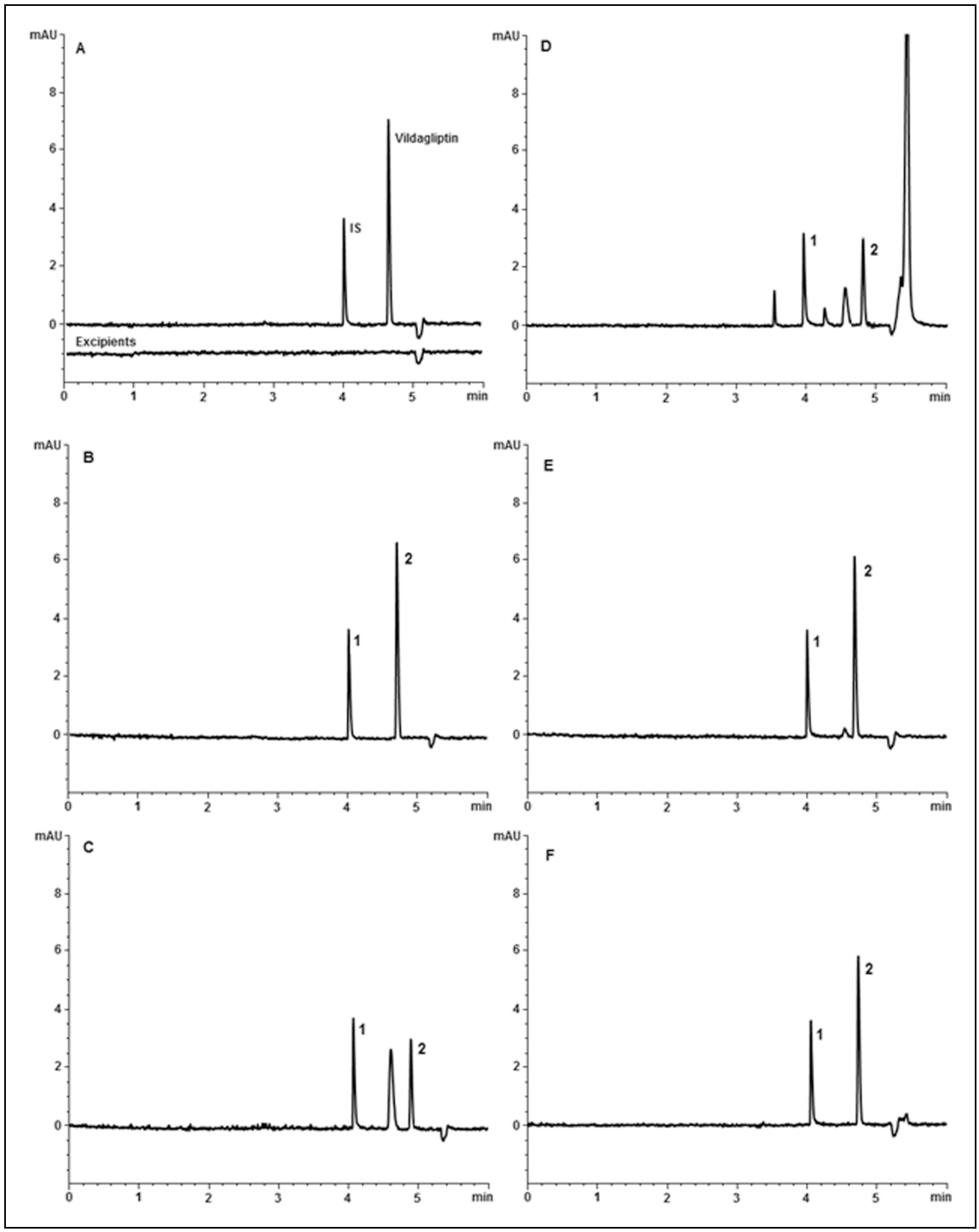

Fig. 2: Typical CZE eletropherograms obtained of vildagliptin drug product (VLG 50 µg/mL). (A) Excipient solution; (B) Acidic condition; (C) Basic condition; (D) Oxidative condition; (E) Thermal condition; (F) Photolytic condition. Peak 1: Ranitidine hydrochloride (IS), Peak 2: VLG. Eletrophoretic conditions: Fused silica capillary (64.5 cm total length and 56.0 cm effective length, 50 µm i.d.), background electrolyte (BGE) consisting of 25 mM potassium phosphate (pH 8.0), voltage of 25 kV (positive polarity), temperature of the capillary cartridge 25 °C. UV/PDA detection at 207 nm.

Table 2: Accuracy values of CZE for vildagliptin in tablet samples

Nominal concentration (µg mL^{-1})	Mean concentration found[a] (µg mL^{-1})	Accuracy (%)	RSD (%)	Bias[b] (%)
25	24.66	98.65	1.88	−1.36
50	49.36	98.71	0.53	−1.28
75	74.65	99.53	0.18	−0.47

[a] Mean of three replicates

[b] Bias = [(measured concentration – nominal concentration)/nominal concentration] x 100

Table 3: Responses obtained by robustness experiment for vildagliptin in Tablets

Experiment	Electrolyte (mM)	pH	Voltage (Kv)	Temperature (°C)	Mean (%)
1	26	7.9	24	26	96.45
2	26	8.1	26	24	99.20
3	24	8.1	24	26	95.57
4	26	7.9	26	26	97.96
5	26	8.1	26	24	97.70
6	26	8.1	24	26	96.18
7	24	8.1	26	26	95.90
8	24	7.9	26	26	98.58
9	24	7.9	26	24	100.47
10	26	7.9	24	24	97.88
11	24	8.1	24	24	95.56
12	24	7.9	24	24	96.14
13	25	8.0	25	25	98.88

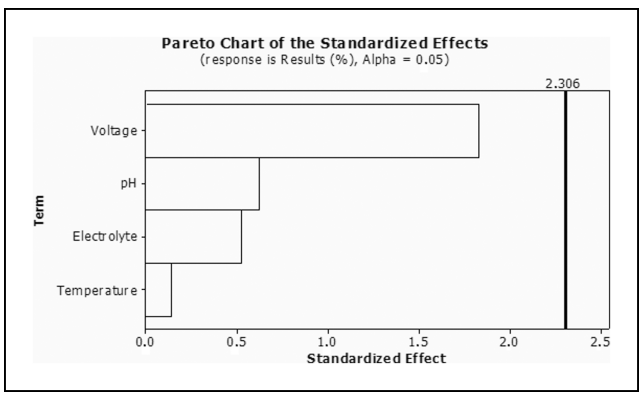

Fig. 3: Pareto chart representing the effects of the variables on the VLG assay for robustness test using Plackett-Burman design.

The validated CZE method was applied for the determination of vildagliptin in pharmaceutical dosage form and the results of precision were compared to those obtained using a validated LC method giving low mean values, as shown in Table 4. The precision of the two methods were compared statistically by Student's *t*-test demonstrating non-significant difference ($p < 0.05$). Therefore, both methods are considered the most widely used methods for analyzing substances of pharmaceutical interest and are both reliable for determination of vildagliptin in tablets.

2.3. Conclusion

A stability-indicating CZE method was successfully developed for vildagliptin in tablet formulation. The results of the validation studies showed that the method was adequate for all parameters tested. The validated method can be useful for the determination of vildagliptin in tablets, such as the validated LC method, with advantages of small sample volumes, employing an aqueous system rather than the potentially toxic organic solvents, short analysis time and may be used as an alternative method in quality control routine analysis.

3. Experimental

3.1. Instrumentation

All the capillary electrophoresis experiments were performed on an Agilent 1000 CE system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector. CE Chemstation® software was used for instrument control, data acquisition and data analysis. Fused silica capillaries (50 μm i.d.) were obtained from Polymicro (Phoenix, USA) and cut to an effective length of 56 cm (total length 64.5 cm). The pH values of the running buffer solutions were adjusted using Digimed pH-meter to within ± 0.02. The BGE solution was prepared and filtered daily.

LC was carried out on an Agilent liquid chromatograph (Santa Clara, CA, United States) equipped with a model Q1311A quaternary pump, ALS-G1329A auto sampler, TCC-G1316A column oven, 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.

3.2. Materials and reagents

The reference standard for VLG (purity of 99.5%) was purchased from Sequoia Research Products (United Kingdom) and the commercial tablets containing 50 mg of VLG (Galvus®, Novartis Biociências S. A., SP, Brazil) were obtained from commercial sources within their shelf life period. All chemicals used were of pharmaceutical or special analytical grade. The reference and sample solutions were filtered through a 0.45 μm membrane filter (Millipore, Bedford, USA). Purified water was obtained by a Millipore® Direct-Q 3UV with pump (Molsheim, AL, France).

3.3. Solutions

3.3.1. Preparation of reference standard solution

Stock solution was prepared by weighing accurately 25 mg of VLG reference substance, transferred to an individual 25 mL volumetric flask and diluted to volume with water, obtaining the concentration of 1.0 mg/mL. The stock solution was stored at 2–8 °C, protected from light. In the work day, the stock solution was diluted to appropriate concentration in purified water and added a constant amount of RH (100 μg/mL) to specific CE individual vials.

3.3.2. Preparation of sample solution

The tablets containing 50 mg of VLG were accurately weighed and crushed to fine powder with a pestle into a mortar. An appropriate amount was transferred into an individual 25 mL volumetric flask, diluted to volume with water, sonicated for 10 min and filtered through a filter paper, obtaining the concentration of 1.0 mg/mL of VLG. This stock solution was stored at 2–8 °C and protected from light. Working sample solutions were prepared by diluting the stock solution to the appropriate concentration and add an amount of RH (final concentration 100 μg/mL) in purified water. Even, sample solutions were filtered through a 0.45 μm membrane filter into specific CE individual vials.

3.4. Analytical conditions

3.4.1. Capillary electrophoresis procedure

All experiments were carried out on a fused-silica capillary with 50 μm i.d. and 64.5 cm of total length (effective length 56 cm), thermostatized at 25 °C, and detection by PDA set at 207 nm. Samples were injected by applying a pressure of 50 mbar for 5 s. A constant voltage of + 25 kV was applied throughout the analysis. Under these conditions, the generated CE

Table 4: Assay results obtained by LC and CZE methods for vildagliptin in a pharmaceutical formulation

	LC method Experimental amount (mg/tablet)	R.S.D. (%)	CZE method Experimental amount (mg/tablet)	R.S.D. (%)
Day 1*	48.74	0.92	48.92	1.03
Day 2*	48.68	0.52	49.43	0.91
Day 3*	49.10	0.37	49.63	0.83
P-value ($P > 0.05$)		*t* calculated = 0.122 < *t* critical = 2.78		

* Mean of six determinations

current was, approximately, 26 μA. The BGE solution consisted of 25 mM potassium phosphate pH 8.0, adjusted by adding sodium hydroxide solution. In the first run, a new capillary was flushed with 1.0 M sodium hydroxide for 30 min, water for 15 min and with buffer solution for 15 min. Between each run the capillary was conditioned with 0.1 M sodium hydroxide for 2 min, water for 1 min and the buffer solution for 2 min successively. The running electrolyte was replaced by a fresh solution after each three injections because of the method reproducibility.

3.4.2. Liquid chromatographic procedure

The previously validated LC method is described (Barden et al. 2012). Briefly, the experiments were performed on an analytical column XBridge^{TM} C_8 (150 mm x 4.6 mm i.d., 5 μm particle size) (Waters, Ireland), at room temperature, using a mobile-phase consisted 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 of VLG.

3.5. Statistical Software

Robustness experimental design, statistical analysis to comparison of the validated methods and of the obtained validation data were performed by a MINITAB® 15 data analysis software system (Minitab Inc, State College, PA, USA).

3.6. Capillary Zone Electrophoresis Method Validation

Following the International Conference on Harmonization (ICH) guidelines (ICH 2003; ICH 2005), the method was validated according of the parameters specificity, linearity and range, precision, accuracy, robustness, stability and system suitability test. Further, stability-indicating capability was demonstrated by performing forced degradation studies.

3.6.1. Specificity

The excipients interference in the pharmaceutical formulation was determined by the injection of a sample containing only the placebo (in-house mixture of the excipients contained in VLG tablets) solution. This solution was prepared the same way than the sample solution to evaluate the absence of interference from formulation excipients on the VLG peak.
Also, the stability-indicating capability of the CZE method was determined by subjecting sample solutions from the pharmaceutical formulation, containing 1.0 mg/mL of VLG, to stress degradation as acid and basic hydrolysis, oxidative, thermal and photolytic degradations. The specificity was established by purity determination of VLG in the stressed samples, using a PDA detector.

3.6.2. Forced degradation studies

In order to evaluate acid and basic hydrolysis, sample solutions (1.0 mg/mL) were prepared and maintained in 0.1 M hydrochloric acid and 0.1 M sodium hydroxide for 4 h in both cases. After the exposition time, the solutions were neutralized with acid or base, as necessary. The oxidative degradation was verified for submit the sample solution to 0.3% hydrogen peroxide, at room temperature, for 2 h. Thermal degradation was evaluated by exposing sample solution, sealed in glass vial, at 60 °C temperature during 48 h. Photodegradation was induced by sample exposition into a chamber to 200 watt hours/square meter of near UV light for 48 h. All the solutions were diluted in water to final concentrations of 100 μg/mL and injected in triplicate.

3.6.3. Linearity

Linearity was obtained by constructing three analytical curves independently each one with seven reference standard concentrations, in the range of 50–200 μg/mL of VLG, prepared with fixed IS concentration (100 μg/mL) in water. Triplicate injections of each reference standard solution were made in order to verify the detector response repeatability. In the obtained electropherograms, the peak areas ratio of vildagliptin reference standard to the IS against the respective reference concentrations were plotted in a graph. The results were subjected by the least square regression analysis to calculate calibration equation and correlation coefficient and by ANOVA (analysis of variance) for compliance of the linear model.

3.6.4. Limits of detection (LOD) and limits of quantitation (LOQ)

LOD and LOQ parameters are useful to demonstrate the sensitivity of an analytical method. The LOD and the LOQ were calculated from the slope and the standard deviation of the intercept of the mean of three calibration curves, determined by a linear regression model, as defined by ICH Guideline.

3.6.5. Precision and accuracy

The precision of the method was determined by repeatability and intermediate precision studies. Repeatability was verified by six independent sample preparations of the same concentration of VLG and IS (100 μg/mL) on the same day, injected in three replicates, under the same experimental conditions. Intermediate precision was assessed by carrying out the analysis on three different days (inter-days) and comparing the results by the RSD determination of the peak area ratio among these days.
The accuracy was determined by adding known amounts of VLG reference substance to the sample solution. From a sample concentration of 1.0 mg/mL, aliquots of this solution were transferred into eppendorf tubes containing vildagliptin standard solution with IS to obtain solutions of 125, 150 and 175 μg/mL, corresponding to 25, 50 and 75% of the nominal analytical concentration (100 μg/mL). The results were expressed as the percentage of VLG reference substance recovered from the sample.

3.6.6. Robustness

Robustness was obtained by evaluating small variations in some electrophoretic parameters such as electrolyte solution pH (7.8 and 8.1), electrolyte concentration (24 and 26 mM), capillary temperature (24 and 26 °C) and voltage applied (24 and 26 kV) in a Plackett-Burman experiment. The VLG analysis, to standard and sample solutions containing 100 μg/mL, was carried out in triplicate ranging all parameters in the same time.

3.6.7. System suitability

The system suitability test was also carried out to evaluate the reproducibility of the system for the analysis to be performed in order to assure that the developed method can be reproduce quality data based on pharmacopeial requirements. Parameters measured in this study were peak area, migration time, theoretical plates, resolution and peak asymmetry.

3.6.8. Comparative Study

The obtained precision results by CZE were compared statistically by Student's *t*-test to those obtained using the validated LC method.

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