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Levodopa microparticles for pulmonary delivery: photodegradation kinetics and LC stability-indicating method

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Levodopa, (*S*)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid, is still considered the gold standard treatment for Parkinson's disease. However, oral levodopa shows poor pharmacokinetics and its efficacy becomes problematic with the progression of the disease. Pulmonary delivery using the association of the polymers: chitosan, hyaluronic acid and HPMC, represents a novel approach to overcome this problem. A stability-indicating liquid chromatography method for the quantitative determination of levodopa microparticles for pulmonary delivery was developed as well as its photodegradation kinetics in solution. The developed and validated method was applied for the analyses of the novel formulation as well as for protocols of stability studies.

1. Introduction

Parkinson's disease (PD) is a chronic and progressive neurodegenerative movement disorder characterized by motor and non-motor symptoms such as poor balance, tremors, rigidity, bradykinesia and difficulty walking (Simuni et al. 2009). The disease has a world wide distribution and it is considered the second most common progressive neurodegenerative disorder, being a leading cause of neurological disability (Shastry 2001; Goole and Amighi 2009).

Exogenous substitution with dopamine's prodrug, levodopa, chemically (*S*)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid (Fig. 1), is the most effective pharmacological treatment for PD. The reason is that PD symptoms are related to a progressive loss of dopamine in the basal ganglia (Fahn 2006; Goole and Amighi 2009).

However, levodopa shows a variable oral bioavailability. Therefore, researchers have been found alternative routes of administration (Nyholm 2006; Goole and Amighi 2009; Kim et al. 2009), for example, the pulmonary route that shows several advantages as no first-pass liver metabolism and fast drug absorption through the alveoli occurs (Jain 2008). Then, a novel spray-dried formulation of levodopa microparticles containing chitosan, hyaluronic acid and hydroxypropyl methylcellulose (HPMC) for pulmonary delivery has been developed focused on the concept of slowing and targeting the release of levodopa to prolong the therapeutic effect, reducing dose and frequency. In a study published by Bartus et al. (2004), it was demonstrated that pulmonary delivery could be a way to improve levodopa bioavailability.

Chitosan and hyaluronic acid are mucoadhesive polymers that act as drug release modifiers and increase pulmonary absorption by delaying mucociliary clearance (Lim et al. 2000; Cook et al. 2005; Learoyd et al. 2008; Sivadas et al. 2008). Indeed, these polymers are biocompatible, biodegradable and show low toxicity (Hirano et al. 1988; Knapczyk et al. 1989; Dornish et al. 1997; Hwang et al. 2008). HPMC was previously used as particle stabilizer and shaper for dry powder inhalers (Steckel et al. 2003; Colombo et al. 2008)

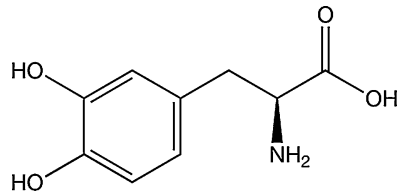

Fig. 1: Levodopa chemical structure

Levodopa microparticles for pulmonary delivery were prepared and characterized in earlier studies. These studies showed adequate results for a pulmonary formulation. Therefore, photodegradation kinetics studies of levodopa microparticles in solution were performed in order to provide evidence on how the quality of the drug varies by the time under the influence of light. To conduct this study, a developed stability-indicating method was applied. A stability-indicating method is necessary to quantify the drug in the presence of its degradation products, and the method should be capable of resolving and detecting photolytic degradants that appear during the study (ICH 2003; ICH 2005). The aim of this study was to develop and validate a stability-indicating LC method for levodopa quantification in the microparticles as well as to determine its photodegradation kinetics in solution.

2. Investigations, results and discussion

2.1. Validation of LC method

First studies were done using levodopa and levodopa tablets LC assay described in the US Pharmacopeia (USP 2009). However,

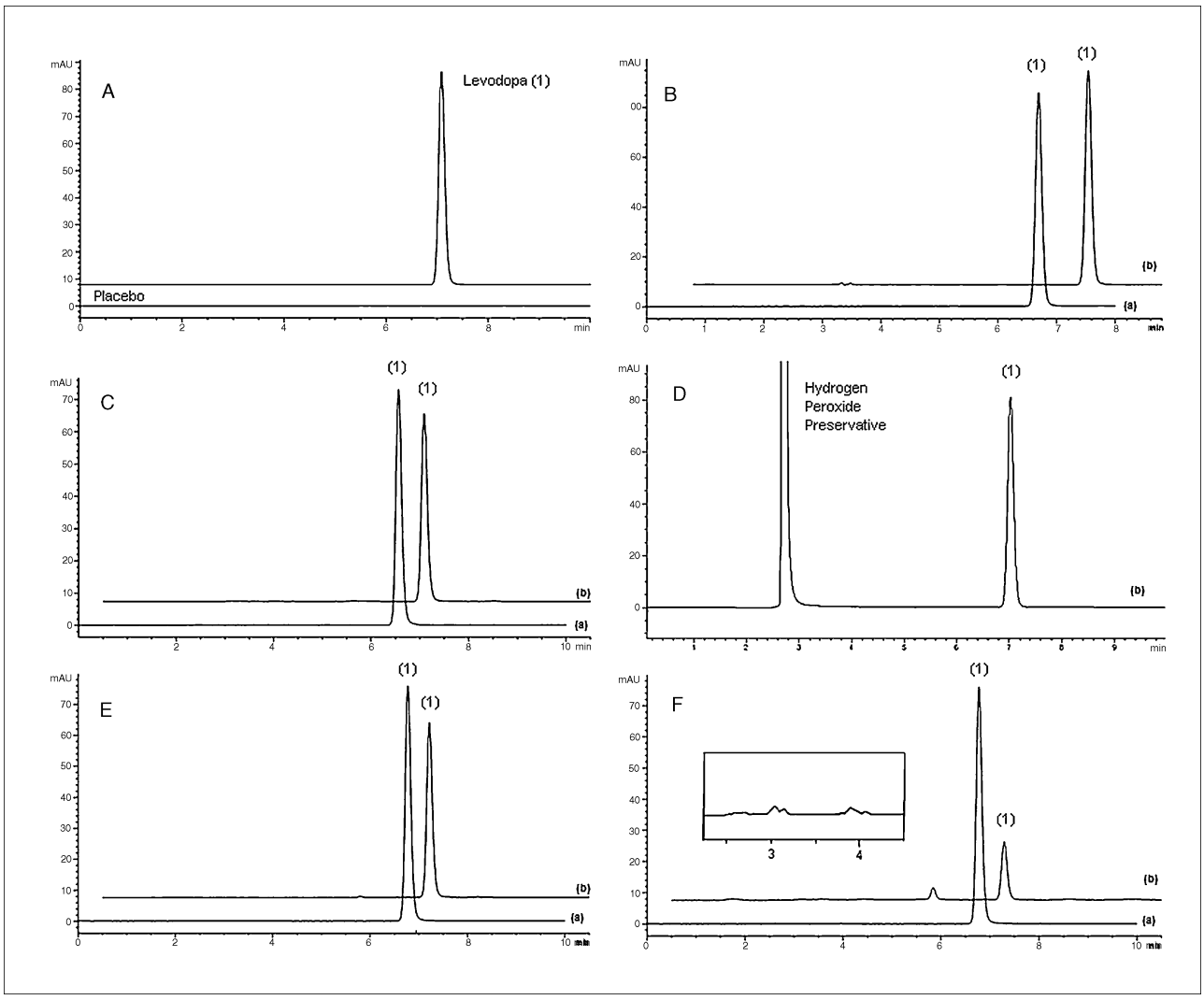

Fig. 2: Chromatograms obtained during specificity study: (A) formulation excipients and reference substance (40 µg mL-1); (B) (a) acid hydrolysis (HCl 0.1 M) and (b) acid hydrolysis (HCl 0.1 M) after 4 days; (C) (a) basic hydrolysis (0.01 M NaOH) and (b) basic hydrolysis (0.01 M NaOH) after 30 min; (D) oxidative degradation (30% H2O2) after 48 h; (E) (a) dry heat 60 °C and (b) dry heat 60 °C for 7 days; (F) (a) photodegradation (UVC) and (b) photodegradation (UVC) after 72 h

the results of system suitability parameters were not adequate and levodopa peak presented an asymmetry due to the high concentration of the standard solution proposed in the assay (0.4 mg/mL). Then, a new method was developed and validated to quantify levodopa in the microparticles formulation.

The new method showed adequate system suitability parameters. The analysis of the levodopa reference standard solution evaluated at each day presented the approximate results: 16.259 theoretical plates, 7.34 resolution between the drug and the main degradation product, 0.85 peak asymmetry or tailing factor and 2.04 for the retention factor. The levodopa RSD area peak of six injections was 0.05%, demonstrating the injection repeatability. According to these results, the LC system and procedure showed that are capable to provide data of acceptance quality (US FDA 1994; Shabir 2003).

Besides that, a stability test was performed with two solutions: one of them containing the reference standard and the other containing the levodopa microparticles, both in mobile phase and prepared according to section 3.2. After 15 h of analysis there was no significant alteration in the measured areas.

2.1.1. Specificity

Placebo injections were performed to demonstrate the absence of excipient components interference with levodopa elution. The results showed that the LC method was not interfered by the formulation excipients, since there was no other peak in the same retention time of levodopa, as presented in Fig. 2.

The sample solutions (levodopa microparticles and standard reference) were submitted to different stress conditions to induce degradation (Fig. 2). Initially, samples were exposed to UV-A radiation but no degradation was observed. Therefore, samples were exposed to UV-C radiation, and after 4 h the samples exhibited an area decrease of 13.71% (levodopa microparticles) and 9.98% for reference standard. Only after 72 h of UV-C light exposure a product degradation peak appeared at a retention time of 5.23 min corresponding to 22.53% of levodopa area peak, other small peaks also appeared around 3 and 4 min, as shown in Fig. 2. These samples presented an area decrease of 70.08% for levodopa microparticles and 57.78% for reference standard in 6.7 min. The results obtained in this preliminary stability study shows that levodopa is susceptible to photodegradation. Probably, the higher degradation of the formulation samples should be caused by the excipients.

When submitted to 60 °C for 7 days, the samples showed an area reduction of 21.83% and 24.23% for the reference standard and levodopa microparticles solution, respectively.

The alkaline degradation in 0.005 M NaOH presented an instantaneous degradation that could be seen as the formation of a yellow color in the solution, which increased along the exposure time. After 30 min, it was observed that 15.83% of the drug degraded in the levodopa microparticles and 14.90% in

the reference standard solution, but there was no corresponding formation of degradation products as compared to the drug standard solution without degradation.

The samples remained stable under acidic conditions, HCl 0.1 M for 4 days. In a study published by Kankkunen et al. (2002), at pH 10.0 the oxidation of levodopa was fast. The oxidation process was slower at pH 7.4 than at pH 10.0. Even after 50 h in the more acidic solutions (pH 2.0 or 4.0), levodopa remained almost completely in an unoxidized form. During levodopa oxidation, the hydrogen atoms come off, therefore, in an acidic environment the oxidation of levodopa is hindered.

The drug was found to be stable in 3% H_2O_2 for 48 h and in 10% H_2O_2 for 60 h at room temperature. After 48 h in oxidation media, H_2O_2 30%, 93.44% of levodopa remained in the levodopa microparticles and 98.45% in the standard solution. This higher degradation in sample solution can be due to the action of one or more excipient ingredients that facilitate the degradation reaction.

Levodopa presents a similar structure to hydroquinone (double-OH-substituted aromatic ring), and hydroquinones can be oxidized to quinones trough a reaction that involves two-one electron transfer (Hovorka 2001). According to Kankkunen et al. (2002), the oxidation process proceeds with very mild oxidizing agents, such as Ag^+ or Fe^{+3} present, e.g. in moisture or water. The oxidation of levodopa can be observed also visually, because the hydroquinones are colorless and the quinones are colored.

For most degradation conditions no degradation peaks were detected. In accordance with Bakshi and Singh (2002), such a situation could be due to drug decomposition into low molecular weight fractions or due to the formation of non-chromophoric products. The chromatographic peak purity tool was applied to all levodopa peaks, and demonstrated that they were pure in all cases, confirming the absence of other substance coeluting in the same retention time. Since the main peak of levodopa was not found attributable to any other substance, the method proves to be a stability indication.

2.1.2. Linearity, LOD and LOQ

The method demonstrated to be linear in the concentration range of 10–60 μg/mL, with a correlation coefficient of 0.9999. The slope and the intercept obtained from the three standard curves analyzed together were 16.0643 and 0.3634, respectively. The analysis of variance revealed that the obtained results correspond to a linear regression and showed no-significative linearity deviation ($\alpha = 5\%$). The intercept was not significantly different from the theoretical zero value, therefore, there is no interference on the validation (Carr and Wahlich 1990). LOQ and LOD were 208.0 and 46.8 ng/mL, respectively.

2.1.3. Precision

Repeatability and intermediate precision results are expressed as relative standard deviations (RSD %). The results are presented in Table 1 for both the repeatability and the intermediate precision. The variability of the results was low with RSD % values less than 2% to intra-day and 0.28% to inter-day, indicating the precision of the developed method.

Table 1: Precision data of levodopa microparticles

	Intra-day precision (%)	RSD (%)
Day 1	101.19	0.27
Day 2	100.80	1.01
Day 3	101.50	0.61
	Inter-day precision (%)	RSD (%)
	101.16	0.28

Table 2: Recovery studies of levodopa (n = 9)

Added concentration (μg/mL)	Mean recovery (%) ± RSD (%)
30	100.22 ± 0.11
40	100.41 ± 0.47
50	100.32 ± 0.11

2.1.4. Accuracy

The data for accuracy was expressed in terms of levodopa percentage recoveries from the known quantities added to the placebo solution. For each level of levodopa concentration three determinations were performed. These results are summarized in Table 2. The mean recovery data were within the range of 100.01 to 100.93% and the mean RSD % was 0.23, satisfying the acceptance criteria for the study.

2.1.5. Robustness

The calculated response factors of the peaks of interest and the performance of the chromatographic system were not influenced by the modified operational parameters.

2.2. Levodopa photodegradation kinetics

The levodopa photodegradation kinetics were investigated under stress conditions. The exposure to light was found to be an important adverse stability factor. The LC method was used for determination of the drug in the degraded samples. The levodopa photodegradation profile was evaluated at different time intervals. The effect of light on the residual concentration of levodopa in degraded samples is shown in Table 3. It was observed that around 30% of the drug degraded in the microparticles solution after 12 h of light exposure; however, there was no corresponding formation of degradation products. The solutions developed a yellow color, which increased along the exposure time. The temperature inside the chamber was always below 32 °C.

Table 3: Effect of light on the residual concentration of levodopa in degraded samples: (A) levodopa reference standard solution and (B) levodopa microparticles solution

Time (h)	Concentration (μg/mL) (A)	Drug content (%) (A)	Concentration (μg/mL) (B)	Drug content (%) (B)
0	40.00	100.00	40.00	100.00
6	33.25	88.12	33.13	82.82
12	29.98	74.94	29.78	74.45
24	27.06	67.65	25.27	63.18
48	21.40	53.51	15.08	37.53
72	16.89	42.22	12.02	29.92

Table 4: Degradation rate constant (k), half-life ($t_{0.5}$) and t_{90} of levodopa and levodopa microparticles solutions

	k ($h^{-1}.mg^{-1}.L$)	$t_{0.5}$ (h)	t_{90} (h)
Levodopa reference standard solution	0.000453	55.16	6.13
Levodopa microparticles solution	0.000838	29.84	3.32

The chromatograms of the placebo solutions did not present any peak. Therefore, there is no excipient influence in the determination of the drug photodegradation kinetics.

Photodegradation kinetics were calculated through the decrease in drug concentration by time. The drug remaining concentration was calculated at each time interval for the three replicates in comparison with the mean concentration of the standard solution.

Through the evaluation of the determination coefficients obtained by plotting the drug concentration (zero-order process), the log (first-order process) and the reciprocal (second-order process) concentration *versus* time, the degradation of levodopa in mobile phase solutions could be better described as second order kinetic for both the reference standard solution and the formulation, under the applied experimental conditions. Therefore, the degradation speed is proportional to either the concentration of a reactant squared, or the concentration product of two reactants (Nudelman 1975). The obtained degradation rate constants (k), half-life ($t_{0.5}$) and t_{90} are described in Table 4.

2.3. Conclusion

A stability-indicating LC method was developed and validated to evaluate the performance of the microparticles containing levodopa for pulmonary delivery. The validation parameters revealed that accuracy, intermediate precision and repeatability are within the general ranges for acceptance. The LC method proposed was also simple, fast, linear, robust and specific and it is a powerful tool to investigate chemical stability of levodopa and levodopa microparticles. The validated method can be successfully applied for the analyses of the novel formulation developed or as an alternative method of analysis for raw material, as well as for protocols of levodopa stability study.

Through the evaluation of the main factors that affect the drug content in solution and through the levodopa degradation kinetics in solution exposed to UV-C light, the method showed its applicability for stability studies. Both solutions of levodopa, reference standard and formulation, were stable under acidic conditions. Mild degradation of the drug occurred under oxidative stress. Levodopa was found to degrade significantly under alkaline conditions, thermal exposure and in the presence of light. The drug kinetics photodegradation was also determined and follows second order reaction kinetics, establishing that its speed is dependent on two factors.

3. Experimental

3.1. Materials

Levodopa (99.99%) was purchased from Henrifarma (São Paulo, Brazil). Chitosan, low molecular weight (LMW: 50 kDa) was sourced from Sigma-Aldrich Chemicals (St. Louis, USA), hyaluronic acid was purchased from Purebulk (Oregon, USA) and Methocel® E3 (HPMC) was kindly donated by Colorcon (Pennsylvania, USA). All other solvents or chemicals were of LC or analytical grade. Purified water was obtained by a Millipore® Direct-Q 3UV (Molsheim, France).

3.2. Levodopa inhalable powder preparation

An acetic acid 0.5% (v/v) solution containing levodopa and the polymers as excipients was prepared. The solution was introduced into a spray-drier (LM-MSD1.0: Labmaq São Paulo, Brazil) using the following operating conditions: inlet temperature, 120 °C; spray flow rate, 0.3 L/h and air pressure, 50 KPa.

3.3. Liquid chromatography (LC) assay of levodopa and levodopa microparticles

Method development, optimization and validation were conducted on an Agilent 1200 series LC (Santa Clara, USA) consisted of a G1311A quaternary pump, G1316A thermostat column compartment, G1329A standard auto sampler, G1322A vacuum degasser and G1315B diode array detector set at 280 nm. The analytical column ACE® RP-18 octadecyl silane (250 × 4.6 mm I.D. particle size 5 μm) was operated with 0.01 M monobasic potassium phosphate adjusted to a pH value 3.0 with phosphoric acid 85% at a flow rate of 1.0 mL/min. The temperature was set at 25 °C in the column oven. The sample injection volume was 20 μL and the run time was 10 min.

3.4. Sample preparation for liquid chromatography analysis

The stock solution of levodopa (0.2 mg/mL) was prepared with mobile phase solution. The working standard solution (40 μg/mL) was also obtained by the dilution of the stock solution in mobile phase.

For the sample solution, a quantity equivalent of 10 mg levodopa was transferred to a 50 mL volumetric flask, and then it was added 15 mL of mobile phase, followed by 20 min in ultrasonic bath and 20 min in mechanical shaker. After that, the volume was completed with mobile phase and an aliquot of 2 mL of this solution was diluted in a 10 mL volumetric flask to give a final concentration of 40 μg/mL. Both microparticles and standard solutions were then filtered through a 0.45 μm membrane filter (Millipore®) prior to the injection.

The stability of both solutions in closed vial was verified for 15 h at room temperature.

3.5. Validation procedure

The method was validated for specificity, linearity, detection and quantification limits, precision (repeatability and intermediate precision), accuracy, robustness and system suitability.

3.5.1. Specificity

The interference of the excipients was evaluated by comparing the chromatograms obtained from the microparticles and the standard solution with those obtained from excipients (chitosan, HPMC and hyaluronic acid). To provide an indication of the method stability and specificity, forced degradation studies were also performed (Bakshi and Singh 2002; ICH 2005).

The accelerated degradation conditions applied were: UV-A and UV-C light, thermal stress (dry heat), acid, basic and oxidant media for both levodopa reference standard and levodopa microparticles solutions. Samples were analyzed against a control sample (with no degradation treatment) and under light protection. In order to demonstrate no interference, excipient solutions were submitted to the same degradation conditions.

Effect of UV-A and UV-C light: 0.5 mL of a solution containing 1 mg/mL of levodopa in mobile phase was placed in a closed 1 cm quartz cell. The cells were exposed to UV chambers (100 × 18 × 17 cm) with internal mirrors and UV fluorescent lamps, one emitting radiation at 352 nm (UV-A) and the other at 254 nm (UV-C). The same procedure was done for the control sample simultaneously, which was wrapped in aluminum foil in order to protect from light. After the degradation treatment, the samples were diluted to 40 μg/mL with mobile phase and immediately analyzed.

Effect of thermal stress: the stock solutions (1 mg/mL) were prepared in mobile phase and kept for 7 days at 60 °C in closed test tubes. A 2.0 mL aliquot of this solution was diluted in mobile phase to a final concentration of 40 μg/mL.

Effect of acid hydrolysis: the samples were dissolved in mobile phase (1 mg/mL) and an aliquot of this solution was diluted in HCl 0.1 M (0.2 mg/mL), allowed to react for 4 days, neutralized with NaOH 0.01 M and diluted in mobile phase to a final concentration of 40 μg/mL.

Effect of alkaline hydrolysis: 20 mL of the stock solution (1 mg/mL) was transferred to a 100 mL volumetric flask and the volume was completed with NaOH 0.005 M. After 30 min, one aliquot of the solution was neutralized with HCl 0.005 M and diluted with mobile phase until the final concentration of 40 μg/mL.

Effect of oxidation: initial studies were performed in 3% H_2O_2 at room temperature for 48 h. Subsequently, the drug was exposed to 10% H_2O_2 for a period of 60 h at room temperature, but no degradation was observed. Finally, samples were dissolved in mobile phase (1 mg/mL) and 5 mL of this solution was transferred to a volumetric flask, where hydrogen peroxide

solution (30%) was added (0.2 mg/mL). After 48 h the solution was diluted until a final concentration of 40 μg/mL, filtered and analyzed.

3.5.2. Linearity

Standard plots were constructed with six drug concentrations in the range of 10–60 μg/mL in triplicates. The linear regression analysis calculated by the least square regression and by variance analysis (ANOVA) was applied to verify the method linearity.

3.5.3. Quantification and detection limits

The quantification (LOQ) and detection (LOD) limits were obtained based on a signal-to-noise approach. After the blank injection, the background noise was observed over a distance equal to 20 times the width at half-height of the peak in a chromatogram obtained by the injection of 40 μg/mL of the reference standard (Carr et al. 1990; Ermer 2005). The signal-to-noise ratio applied was 10:1 for the LOQ and 3:1 for the LOD. The results were verified experimentally.

3.5.4. Precision

The precision determination was done by analyzing six sample solutions of levodopa microparticles in the same day for intra-day precision (repeatability) and on three different days for inter-day precision (intermediate precision). The relative standard deviation (RSD %) was determined.

3.5.5. Accuracy

The accuracy was determined by the recovery of known amounts of levodopa reference standard added to the placebo solution. The added levels were 75, 100 and 125% of the nominal drug concentration (40 μg/mL). The results were expressed as the percentage of levodopa reference standard recovered.

3.5.6. Robustness

The robustness of the analytical method was performed in order to evaluate the susceptibility of measurements due to deliberate variations in analytical conditions. It was determined by analyzing the standard and sample solutions with the following deliberate changes to the chromatographic conditions: column temperature ± 5 °C, flow rate ± 0.2 mL/min, mobile phase pH ± 0.2 unit variation and column of a different serial number.

3.5.7. System suitability

During different days of the method validation, the system suitability was evaluated through the obtained parameters for the standard solution, such as theoretical plates, peak asymmetry, resolution and retention factor. The injection precision was calculated according to US Pharmacopeia (2009) and the LC software calculated the resolution between the drug and the main photodegradation product.

3.6. Levodopa photodegradation kinetics

The photodegradation kinetics were investigated with quartz cells containing 0.5 mL of levodopa in mobile phase at a concentration of 0.1 mg/mL exposed to UV-C radiation (254 nm). The experimental conditions for the drug irradiation were the same as described for the specificity analysis under UV light in section 3.5.1. At pre-established times (0, 12, 24, 48 and 72 h), the solutions (n = 3) were diluted in mobile phase to achieve the final theoretical concentration of 40 μg/mL. Using the developed and validated method, these solutions were analyzed by LC.

Protected samples, wrapped in aluminum foil, were exposed to the conditions described above to evaluate the contribution of thermally induced degradation. The temperature inside the chamber was controlled during the experiment.

By plotting the drug concentration (zero-order process), the log (first-order process) and the reciprocal (second-order process) concentration *versus* time, the levodopa photodegradation kinetic rate was determined. The reaction order was set through the best observed fit of the obtained determination coefficients (R^2). The kinetic parameters such as apparent order degradation rate constant (k), half-life ($t_{0.5}$) and t_{90} (i.e. time were 90% of original concentration of the drug is left unchanged) were calculated. Each experiment was done in triplicate (analysis by LC method).

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