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Photodegradation kinetics, cytotoxicity assay and determination by stability-indicating HPLC method of mianserin hydrochloride

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A stability-indicating HPLC method for the determination of mianserin hydrochloride in coated tablets was developed and validated. Also, drug photodegradation kinetics and cytotoxicity were determined. Chromatographic analyses were performed in an Ace RP-18 octadecyl silane column (250 mm × 4.6 mm i.d., particle size 5 μm) maintained at ambient temperature (25 °C). The mobile phase was composed of methanol, 50 mM monobasic potassium phosphate buffer and 0.3% triethylamine solution adjusted to pH 7.0 with phosphoric acid 10% (85:15, v/v) in isocratic mode at a flow rate of 1.0 mL·min^{-1}. The performed degradation conditions were: acid and basic media with HCl 1.0 M and NaOH 1.0 M, respectively, oxidation with H_2O_2 3% and the exposure to UV-C light. No interference in the mianserin hydrochloride elution was verified by degradation products formed. Linearity was assessed and ANOVA showed non-significant linearity deviation ($p > 0.05$). Adequate results were obtained for repeatability, intermediate precision, accuracy and robustness. The photodegradation kinetics of mianserin hydrochloride was evaluated in methanol. The degradation of mianserin could be better described as zero order kinetic (r = 0.9982). The UV-C degraded samples of mianserin hydrochloride were also studied in order to determine the preliminary cytotoxicity *in vitro* against mononuclear cells.

1. Introduction

Mianserin hydrochloride (Fig. 1) is a drug for the treatment of depressive illness and depression associated with anxiety. Its antidepressant effect is mainly attributed to presynaptic alfa2-adrenoreceptor blocking activity and to serotonin receptors antagonism. Mianserin is classified as an atypical antidepressant, based on its mechanism of action not totally defined (Pawlowska et al. 2003).

It is administered as a racemate of R-(−) and S-(+)-mianserin hydrochloride in a dose of 30–90 mg/day. It takes 2–3 h to reach the peak concentration of mianserin after oral administration, the elimination half-life is 14–33 h which is prolonged in the elderly (The Merck Index 1996).

The literature reports the quantitative determination of mianserin in biological fluids by high performance liquid chromatography (HPLC) (Chauhan et al. 2005; Hefnawy et al. 2004; Xu et al. 2008), capillary electrophoresis (Grodner and Pachecka 2006) and spectrophotometric methods with detection in the visible region, based on the formation of complexes (Khan et al. 2002). However, there is no study describing a suitable analytical method that is stability indicating for mianserin hydrochloride in bulk and coated tablets, to quantify the drug in the presence of its degradation products.

The lack of reliable methods for the quality assessment of pharmaceutical products limits the efficiency and monitoring of the validation programs (Swartz and Krull 1998). Thus, only the validation of analytical methods, used to verify the quality of drugs, ensures that they meet the requirements of analytical applications and ensure the reliability of the results (BRASIL 2003; ICH 2005; USP 32, 2009).

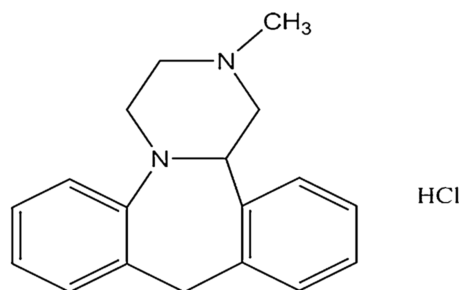

Fig. 1: Chemical structure of mianserin hydrochloride (M1)

According to ICH (ICH 2003), stress testing of the drug substance can help to identify the likely degradation products, which can cooperate to establish the degradation pathways and the molecule intrinsic stability. In agreement with this guide, the light testing should be an integral part of the stress testing.

The aim of this study was to develop and validate a stability-indicating HPLC method in compliance with the ICH Guideline and the United States Pharmacopoeia (USP) for the determination of mianserin hydrochloride in tablets as well as to determine the photodegradation kinetics of the drug in methanolic solutions and cytotoxicity of photodegradation products.

Table 1: Precision of HPLC method to determination of mianserin hydrochloride

	Intra-day precision (%), n = 6	RSD (%)
Day 1	99.24	0.39
Day 2	99.93	0.44
Day 3	101.03	0.23
	Inter-day precision (%), n = 18	RSD (%)
	100.07	0.83

Table 2: Accuracy of HPLC method to determination of mianserin hydrochloride

Added concentration (μg.mL^{-1})	Mean concentration found (μg.mL^{-1})	Mean recovery (%) ± RSD%
12.5	12.91	103.28 ± 0.83
25.0	25.60	102.40 ± 0.76
37.5	37.79	100.77 ± 0.45

2. Investigations, results and discussion

The chromatographic conditions were adjusted in order to provide a good performance of the assay. During the optimization of the method, different columns and organic solvents, aqueous phase with or without buffer and five different pH values (3.0–7.0) were tested. Mobile phase and column selection were based on peak parameters (tailing factor and theoretical plates), run time, easy preparation and cost. After testing various combinations of mobile phase, the best condition with a retention time of 6.6 min for mianserin, were obtained using an Ace RP-18 octadecyl silane column (250 mm × 4.6 mm i.d., particle size 5 μm) maintained at ambient temperature (25 °C) and the mobile phase was composed of methanol and 50 mM potassium monobasic phosphate buffer with 0.3% triethylamine adjusted to pH 7.0 with phosphoric acid at 10% (85:15, v/v). The addition of triethylamine improved peak symmetry. The retention time observed allows for a rapid determination of the drug. Due to its satisfactory solubility, methanol was used as diluent to dissolve the samples. The observed value of tailing factor (1.18) was below the specified limit (≤ 2).

Mianserin hydrochloride solutions were submitted to different stress conditions to induce degradation (Fig. 2). Under UV-C radiation (254 nm), after 30 min mianserin hydrochloride content exhibited some decrease with additional peaks detected and 50% degradation was observed after 90 min. When acid and basic degradation was performed, no degradation peaks were detected and the concentration of mianserin hydrochloride remained constant during the exposure time to the degradation condition. The oxidation by H_2O_2 caused the degradation of mianserin hydrochloride and one major degradation product peak could be observed. After 6 h, 30% degradation was observed. The described HPLC method is considered specific for mianserin hydrochloride as the peak is clearly separated from the others and the peak of the drug was shown to be pure under all conditions of forced degradation.

The HPLC method was linear (r = 0.9999) at concentrations ranging from 50.0 to 110.0 μg·mL^{-1}. The slope and the intercept obtained from the three standard curves analyzed together were 9.487 and 0.9206, respectively. The intercept was not significantly different from the theoretical zero value ($p > 0.05$) therefore, there is no interference on the validation (Carr and Wahlich 1990). According to ANOVA there is linear regression ($p < 0.05$) and there is no deviation from linearity ($p > 0.05$). The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and it was expressed as RSD (%). The experimental values obtained for the determination of mianserin hydrochloride in samples are presented in Table 1. The low values of relative standard deviations (RSD) for the repeatability (0.39%, 0.44% and 0.23%) and intermediate precision (0.83%) demonstrated adequate precision of the analytical method. The accuracy of the method was determined and the mean recovery was found to be 102.15% (Table 2). The method was robust when was investigated the influence of temperature, wavelength, organic phase, flow rate and pH (Table 3). The LD and LQ were 0.21 and 1.05 μg·mL^{-1} respectively, demonstrating the sensitivity of the method. The sample solutions stored at 4 °C remained stable for up to seven days, while solutions kept at room temperature remained stable for 24 h.

The purpose of photostability testing is to provide evidence on how the quality of a drug varies with the time under the influence of UV-C light. In this study, mianserin hydrochloride photodegradation kinetics was carried out through the employment of stress conditions.

The mianserin hydrochloride photodegradation kinetic rate was determined 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 mianserin in methanolic solutions could be better described as zero order kinetic (r = 0.9982), where the reaction speed is independent of the concentration of active substance. The obtained degradation rate constants (k) and $t_{0.5}$ were: 0.279 μg·mL^{-1}/min and 143.37 min (Fig. 3).

The cytotoxicity assay against mononuclear cells was developed in order to evaluate the probable cytotoxic potential of UV-C light degraded samples. It was performed to evaluate the effect of the degraded structures in relation to the intact molecule. This test allows predicting possible adverse effects resulting from degraded samples. Triton X-100 1% was used as a parameter because it causes 100% cell death. As shown in Fig. 4, mianserin hydrochloride degraded 200.0 and 500.0 μg·mL^{-1} showed a significant increase in cytosolic LDH release into the incubation medium. Statistical analysis demonstrated that the degraded samples of Mi 500.0 and Mi 200.0 μg·mL^{-1} showed potential cytotoxic effects, since they are significantly different from control and lower concentrations of the solutions. These results

Table 3: Robustness of the HPLC method

Variable	Range investigated	Mianserin % (RSD %)	Tailing factor	Optimized value
Temperature (°C)	23.0	99.84 (0.15%)	1.22	25.0
	27.0	99.96 (0.18%)	1.23	
Wavelength (nm)	276.0	100.09 (0.24%)	1.23	278.0
	280.0	100.22 (0.27%)	1.23	
% Organic phase	83.0	100.06 (0.54%)	1.22	85.0
	87.0	100.19 (0.51%)	1.24	
Flow rate (mL.min^{-1})	0.8	99.85 (0.35%)	1.19	1.0
	1.2	99.93 (0.31%)	1.15	
pH	6.8	100.02 (0.38%)	1.20	7.0
	7.2	100.15 (0.34%)	1.20	

Each sample was injected three times. R.S.D. are listed in brackets.

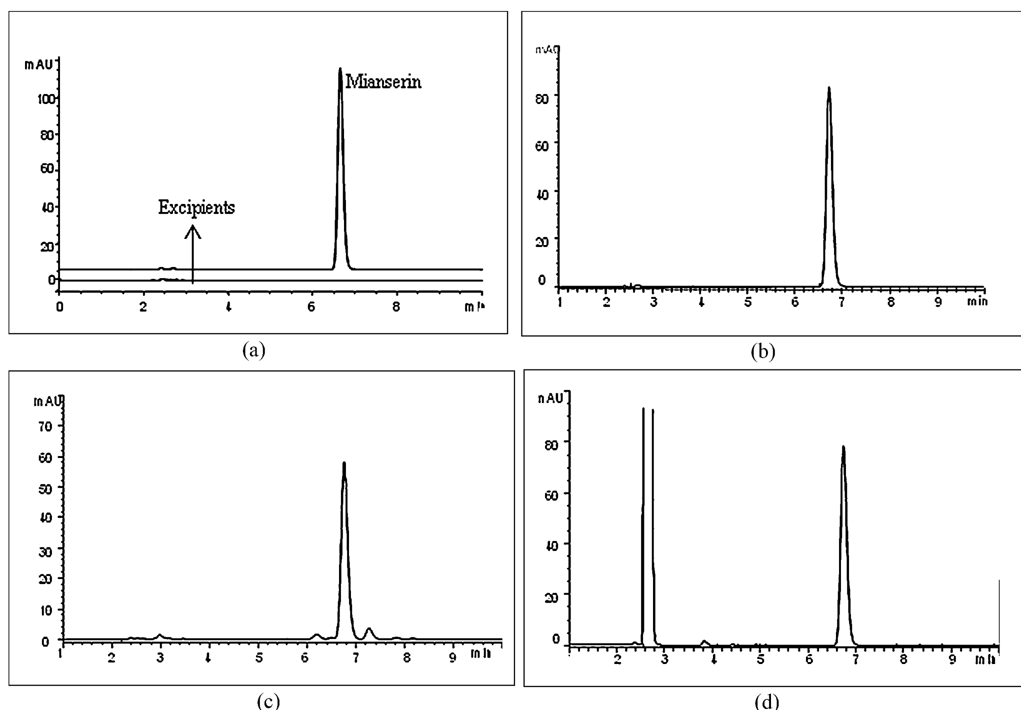

Fig. 2: Chromatograms of the sample and excipients solution (a), after acid - HCl 1.0 M /2 h and basic degradation - NaOH 1.0 M /2 h (b), after UV-C light degradation - 254 nm/30 min (c) and oxidation (d)

indicate that the presence of high concentrations of mianserin hydrochloride degraded samples causes cellular damage, affecting cell integrity. Results are expressed as mean ± standard error for two independent experiments performed in triplicate.

3. Experimental

3.1. Materials and reagents

Mianserin hydrochloride reference standard (99.90%) was kindly supplied by Pharmaceutical Industry Organon (São Paulo, Brazil) and the commercial tablets Tolvon® were obtained from the local market. Purified water was obtained by a Millipore® Direct-Q 3UV with pump (Molsheim, France). HPLC grade methanol, sodium hydroxide, hydrochloric acid, triethylamine, hydrogen peroxide and potassium monobasic phosphate (reagent grade) were purchased from Merck (Darmstadt, Germany). Mianserin hydrochloride coated tablets were claimed to contain 30 mg of the drug and the following inactive ingredients: starch, silicon dioxide, magnesium stearate, methylcellulose, calcium dibasic phosphate, hypromellose, macrogol and titanium dioxide. All the solutions were prepared daily.

3.2. Instrumentation and analytical conditions

The HPLC system (Agilent 1200 series, Santa Clara, USA) consisted of a G1311A quaternary pump, G1322A vacuum degasser, G1316A thermostat column compartment, G1329A standard auto sampler and G1315B diode array detector set at 278 nm. Chromatographic analyses were performed in an Ace RP-18 octadecyl silane column (250 mm × 4.6 mm i.d., particle size 5 μm) maintained at ambient temperature (25 °C). The mobile phase was composed of methanol, monobasic potassium phosphate buffer (50 mM) and 0.3% triethylamine solution adjusted to pH 7.0 with phosphoric acid 10% (85:15, v/v) in isocratic mode at a flow rate of 1.0 mL. min^{-1} and the sample injection volume was 20 μL. The degradation process was carried out with quartz cells containing mianserin in methanolic solution exposed to UV-C radiation (254 nm). The cells were exposed to an UV chamber (100 × 18 × 17 cm) with internal mirrors and UV fluorescent light CSR F30W T8. For cytotoxicity assay a Ficoll-Paque gradient centrifuge (Sigma), Hank's medium, centrifuge, hemocytometer ABX-Micros 60, commercial kit (Doles reagents, Goiânia, Brazil) and a plate reader Envision (Perkin Elmer) were used.

3.3. Sample preparation for HPLC analysis

The stock solution of mianserin hydrochloride reference standard (500.0 $\mu g \cdot mL^{-1}$) was prepared in methanol since the drug is freely soluble in this solvent. The working standard solution (80.0 $\mu g \cdot mL^{-1}$) was obtained by diluting the stock solution in the mobile phase. Twenty tablets were weighed and the average weight was calculated. Tablets were crushed to a fine powder. A quantity of the powdered tablets, equivalent to 4.0 mg of mianserin hydrochloride, was transferred to 25 mL volumetric flasks. Then, 15 mL of methanol was added, followed by 20 min in ultrasonic bath and more methanol was added until the solution reached 160.0 $\mu g \cdot mL^{-1}$. This solution was filtered and one aliquot of the filtrated fluid was diluted with the mobile phase until the final concentration of 80.0 $\mu g \cdot mL^{-1}$. The solution was filtered through a 0.45 μm membrane filter (Millipore).

3.4. Validation of HPLC method

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

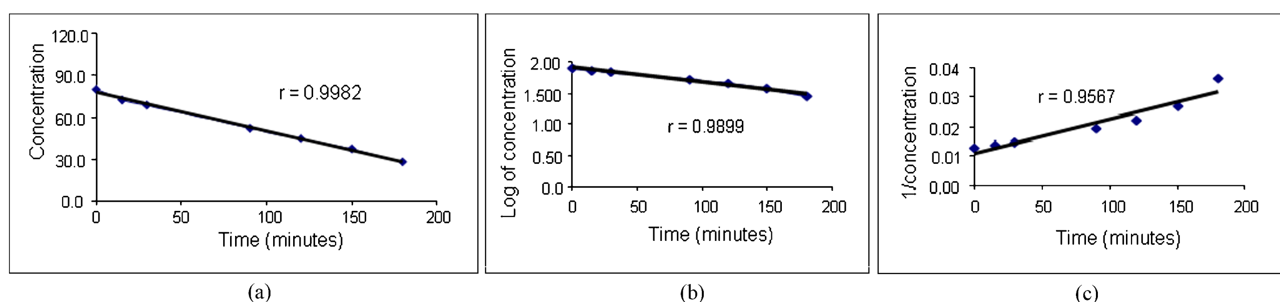

Fig. 3: Plots of concentration (a) zero-order reaction, log of concentration (b) first-order reaction, and reciprocal of concentration (c) second-order reaction of mianserin hydrochloride remaining *versus* time

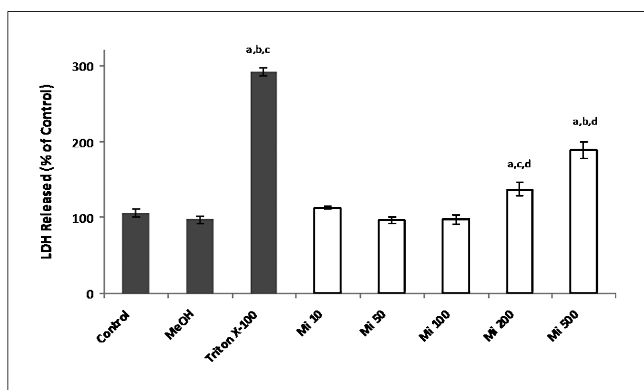

Fig. 4: Effects of different concentrations of mianserin (10–500 μg·mL^{-1}) on cellular integrity, accessed by LDH released. Results are expressed as mean ± S.E.M. for two independent experiments performed in triplicate. Filled bars represent the control groups (methanol 8% and Triton X-100 1%) and open bars represent groups treated with mianserin: [a]values significantly different when compared with the control group; [b]values significantly different when compared with Mi 200; [c]values significantly different when compared with Mi 500; [d]values significantly different when compared with Triton X-100 1%, as determined by one-way ANOVA followed by Duncan's test

Chromatographic runs of a placebo solution and forced degradation studies were performed in order to provide an indication of stability. The stress conditions used were: light, heat, oxidant media acid and base. Samples were analyzed against a freshly prepared control sample (with no degradation treatment) and under light protection. All solutions were injected in triplicate. The peak purity was determined using the Agilent Chemstation software tools. Specific details of the experiments conditions are described below:

Effect of UV light: 1.0 mL of a solution containing 0.4 mg·mL^{-1} of mianserin hydrochloride in methanol was placed in a closed 1.0 cm quartz cells. The cells were exposed to a UV chamber (100 × 18 × 17 cm) with internal mirrors and UV fluorescent light CSR F30W T8 emitting radiation at 254 nm for 15, 30, 45, 60, 90 and 120 min. Protected samples, wrapped in aluminum foil (in order to protect them from light) were submitted simultaneously to identical conditions and used as control. After the degradation treatment, the samples were diluted to 80.0 μg·mL^{-1} with mobile phase and immediately analyzed.

Effect of heat: An amount of powder equivalent to 20.0 mg mianserin hydrochloride was transferred to a 20 mL volumetric amber flask with methanol (1.0 mg·mL^{-1}). The sample was kept at 60 °C for 1, 2, 4 and 6 h to study the effect of the heat. After the specified time, this solution was diluted to 80.0 μg·mL^{-1} with mobile phase.

Effect of oxidation: Mianserin hydrochloride was dissolved in methanol (0.8 mg·mL^{-1}) and 25 mL of this solution were transferred to a volumetric flask (50 mL), where 5.0 mL of hydrogen peroxide solution (30%) was added and the volume was completed with methanol. After 2, 4, 6 and 24 h the solution was diluted until the final concentration of 80.0 μg·mL^{-1}, filtered and analyzed.

Effect of acid and alkaline hydrolysis: 25 mL of the mianserin hydrochloride solution were transferred to a volumetric flask and HCl (acid degradation) or NaOH (alkaline degradation) was added until the final concentration of 1.0 M in both cases. After 2, 4, 6, 8, 12 and 24 h (acid degradation) and 2, 4, 6, 8, 12 and 24 h (basic degradation), one aliquot of the solution was neutralized by NaOH 1.0 M (acid degradation) or with HCl 1.0 M (alkaline degradation) and diluted with mobile phase until the final concentration of 80.0 μg·mL^{-1}.

Appropriate amounts of mianserin hydrochloride reference standard stock solution (500.0 μg·mL^{-1}) were diluted with mobile phase to give a concentrations of 50.0, 60.0, 70.0, 80.0, 90.0, 100.0 and 110.0 μg·mL^{-1}. On three different days each concentration was injected in triplicate and calibration plots were prepared. Linearity was evaluated by linear least-squares regression analysis.

The quantification and detection limits were obtained based on signal-to-noise approach. The background noise was obtained after injection of the blank, observed over a distance equal to 20 times the width at half-height of the peak in a chromatogram obtained by the injection of 80.0 μg·mL^{-1} of the reference standard (Carr and Wahlich 1990; Ermer and Ploss 2005). The signal-to-noise ratio applied was 10:1 for the LOQ and 3:1 for the LOD. The results were verified experimentally.

The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying six samples, at the same concentration (80.0 μg·mL^{-1}) and during the same day. The intermediate precision was studied by comparing the assays on different days (3 days).

The accuracy was determined by the recovery of known amounts of mianserin hydrochloride reference standard added to the samples in the beginning of the preparative process. The added levels were 25, 50 and 75% of the 50.0 μg·mL^{-1} drug concentration. The results were expressed as the percentage of mianserin hydrochloride reference standard recovered from the sample.

Robustness was evaluated by small variations in the pH values of the aqueous phase of mobile phase, column temperature, flow rate, wavelength as well as ratio of the mobile phase. The method robustness was assessed by deliberately modifying the operating conditions of the former and measuring chromatographic parameters such as retention time and tailing factor.

Stability of mianserin hydrochloride in methanolic solution was studied by HPLC method. Sample solutions of mianserin hydrochloride (80.0 μg·mL^{-1}) were prepared in triplicate and stored at 4 and 25 °C for 24 h, 48 h, and 7 days. The stability of these solutions was studied by performing the experiment and looking for the change in the chromatographic pattern compared with freshly prepared solutions.

3.5. Photodegradation kinetics

The exposure to UV-C light was found to be an important stability factor, demonstrated by the specificity of HPLC method. The kinetics of photodegradation of mianserin hydrochloride was evaluated in methanol. A stock solution (400.0 μg·mL^{-1}) was prepared from coated tablets. The stress degradation study was performed exposing the solutions contained in quartz cells in the chamber. The samples were positioned horizontally, to provide the maximum area of exposure to the light source. The irradiation was carried out at 254 nm at different time intervals (0, 15, 30, 45, 60, 90, 120, 150 and 180 min). In order to evaluate the contribution of thermally induced change to the total change, protected samples, wrapped in aluminium foil, were used as dark controls. After each time intervals, the samples were diluted with the mobile phase to give a final theoretical concentration of 80.0 μg·mL^{-1}. The samples were assayed by HPLC. All the solutions were injected in triplicate.

3.6. Cytotoxicity assay

Human mononuclear cells were separated from the peripheral blood of three healthy donors. Heparinized venous blood was diluted 1:1 with Hank's medium. Mononuclear cells were isolated by plasma separation on Ficoll-Paque gradient and washed twice in Hank's medium. The total cells were counted in hemocytometer ABX-Micros 60. Mononuclear cells were washed and resuspended in Hank's medium to a concentration of 10^6 viable cells in 1.0 mL. Pipetting the reagents into a 96-well plate was done in the following order: ferric alum, substrate and sample solution. After addition of the samples, the plates were cultivated in an incubator at 37 °C. Concurrently, a blank test was performed in triplicate with: ferric alum and substrate (the sample was not added at this point). A positive control assay was also performed with the maximum amount of methanol used to prepare the samples and the Triton X-100 (100% cell death). After the sample addition, the plates were incubated at 37 °C. The blank test, the control assay and the sample tests were simultaneously prepared. After this procedure, at each 10 s, stabilization solution was added. The reading of the blank is subtracted from the readings obtained for each sample. In each well plate the final concentration analyzed of each sample was 10.0, 50.0, 100.0, 200.0 and 500.0 μg·mL^{-1}, in triplicate.

The samples were prepared in methanolic solutions and diluted in Hank's medium. The photodegraded solution was prepared by exposing mianserin in methanolic solution (5.0 mg·mL^{-1}) to UV-C radiation (254 nm) for 90 min. After the period of exposition, the solution was diluted with Hank's until final concentration.

Cellular injury was quantified by measuring lactate dehydrogenase (LDH) released into the medium. LDH is a cytosolic enzyme that is released into the cytoplasm upon membrane integrity loss. The activity was determined using a commercial kit (Doles Reagents, Goiânia, Brazil). LDH activity was evaluated using a colorimetric assay at 492 nm. Data from the experiments were analyzed statistically by one-way ANOVA followed by the Duncan's multiple range test when the *F* test was significant. All analyses were performed using Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant, if $p < 0.05$.

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