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Selective reversed-phase liquid chromatography method for the kinetic investigation of 3-hydroxyflavone photostability

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Abstract

This paper reports a fast and accurate RP-HPLC chromatographic method for the simultaneous determination of 3-hydroxyflavone (3-OH F) and its photodegradation products. Solutions (5×10^{-5} M) in acetonitrile (ACN) of the molecule were subjected to forced degradation by exposure to artificial UV-A light source (black-light, λ_{max} 354 nm) and the changes appearing in chromatograms were monitored at selected irradiation times. A multistep gradient was optimised to achieve complete elution of all photoproducts in the shortest analysis time. UV spectra recorded by the diode array detector system (285 and 340 nm) clearly showed the structural changes in the new species formed, with respect to the parent compound. The analytical method was subjected to a validation procedure in which linearity and range, as well as specificity, precision and accuracy were determined according to ICH guidelines. Quantitative evaluation of the photochemical process was performed on the basis of the calculated kinetic parameters: photodegradation rate constant k, half-life time $t_{0.5}$, time degradation of 10% of the drug $t_{0.1}$.

Keywords: Kinetics; Photostability; Validation; 3-Hydroxyflavone

1. Introduction

Photochemical stability of pharmaceutical substances is a matter of great interest, both for analytical and for practical purposes. Drug light-induced degradation can result in a decreased efficacy and sometimes also involve significant adverse side effects after drug administration.

The latter arise from the formation of phototoxic transient or labile species, such as free-radicals and photoproducts; sometimes singlet-oxygen is involved, as a result of energy-transfer reactions [1,2]. An increasing number of drugs belonging to different therapeutic classes (calcium channel blockers, non-steroidal anti-inflammatory drugs, chemotherapeutic agents, diuretics, benzodiazepynes, beta-blockers, etc.) were found to be photolabile, consequently light protection is prescribed for their storage by the pharmacopoeias [3,4].

Flavonoids are involved in electron-transfer processes in biological systems [5] and have been widely investigated as prototype molecules for studying the dynamics of proton-transfer reactions in both excited and ground states [6,7]. Also they are widely used as therapeutic agents in a large variety of diseases and in cosmetic formulations for the prevention of photoaging [8–11].

Among this class of natural compounds, flavonols are known to be the most reactive ones, mainly because of the presence of the free 3-OH group [12–15].

The present work was aimed to investigate the photostability of 3-OH F (Fig. 1) in solution, under forced exposure to UV-A radiation. A selective and reliable liquid chromatographic method (HPLC) was developed and validated according to ICH, for the quantitative evaluation of the drug photodegradation process, in the presence of its photoproducts.

Kinetic parameters of concern in pharmaceutics were afterwards estimated.

2. Experimental

2.1. Chemicals

3-Hydroxyflavone ($C_{15}H_{10}O_3$, M_W = 238.2) was purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile (ACN) and methanol (MeOH), both high-performance

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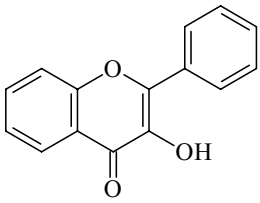

Fig. 1. 3-OH-flavone.

liquid chromatography (HPLC) grade were provided from Merck (Darmstadt, Germany).

Acetic acid glacial for analysis was furnished from Carlo Erba (Milan, Italy).

Water was distilled, deionised and filtered through 0.22 μm Millipore® GSWP filters (Bedford, USA). All solutions investigated in the separations were filtered through 0.45 μm PVDF Whatman® filters (Clifton, NJ).

2.2. Apparatus

Photochemical experiments were carried out using a multi-lamp photoreactor (model MLU 18, Applied Photophysics, London, UK) provided with a six twin-lamp modules arrangement of UV-A black-light (prominent spectral emission at 354 nm).

Light output of the lamps measured with the ferrioxalate actinometer was 2.1×10^{18} photons s^{-1}. Solutions to be irradiated were placed in a round-bottom quartz vessel (34 mm × 360 mm, NS joint 14/23).

The HPLC analyses were performed using a Merck Hitachi (Tokyo, Japan) LaChrom L-7100 pump, equipped with a Rheodyne model 7125-075 injector with a 6 μl sample loop. The eluates were monitored by a Merck Hitachi (Tokyo, Japan) La Chrom-7400 UV detector.

The wavelength was set at 340 nm (λ_{max} of 3-OH F). Integration of the peak areas was done by a Merck Hitachi (Tokyo, Japan) D-7000 HPLC system manager.

The chromatographic separations were performed on a RP-18 Perkin-Elmer (125 mm × 4.6 mm i.d.; 5 μm particle size) column thermostated with a Merck Hitachi L-7300 column oven (25 °C). In order to gain more detailed information about the new species obtained during photolysis, analyses were repeated using a Perkin-Elmer (Norwalk, Connecticut) LC 235 diode array detector (DAD), connected to a Perkin-Elmer (Norwalk, Connecticut) LCI-100 Laboratory Computing Integrator and to a Perkin-Elmer (Norwalk, Connecticut) Series 410 pump, equipped with a Rheodyne model 7125-075 injector with a 6 μl sample loop. Detector wavelength was set at 285 and 340 nm, and an attenuation of 0.05 a.u.f.s. was used. pH values of the analytes were obtained by a Jenway 3310 pHMeter (±0.1).

2.3. Photostability testing

2.3.1. Photolysis

Unbuffered 3-OH F (5×10^{-5} M) in acetonitrile (pH 5.3) were exposed to radiation for prefixed times and monitored by HPLC to evaluate the compound photodegradation. Irradiation was performed in oxygen-saturated solution, magnetically stirred to ensure a homogenous exposure to artificial light. A cooling fan mounted on the photoreactor's base plate avoided overheating of the samples. A vessel containing the same solution was covered with aluminium foil before exposure to serve as a blank.

2.3.2. Analysis of the photoproduct

After selected irradiation times, samples were filtered and analysed by reversed-phase HPLC.

Multistep gradient programme optimised to achieve complete elution of all the photoproducts was MeOH/H_2O (with 10% acetic acid glacial) 30/70 (v/v) 0 min; concave gradient 90/10 (v/v) 25 min; linear gradient 30/70 (v/v) 5 min. The flow rate was 1 ml/min.

Absorption spectra relevant to 3-OH F and its photoproducts were recorded by DAD for monitoring the changes in structures with respect to the parent compound.

3. Results and discussion

3.1. Photodegradation studies

The experimental work was focused on the separation of the photoproducts derived from 3-OH F upon UV-A light-induced degradation.

Solutions of 3-OH F (5×10^{-5} M) in ACN were exposed to UV-A light source and analysed at different irradiation times for monitoring both the degradation of the parent compound and the formation of new species arising from light-induced reactions.

Such a low concentration of the solution to be irradiated was chosen to avoid both intermolecular H-bonding and inn-filter effect caused by the external molecules, which could compromise homogeneous exposure of the samples.

RP-HPLC seems to be the most suitable analytical technique to achieve both qualitative and quantitative information on the photochemical reaction.

Before irradiation, the solution containing pure 3-OH F eluted at a retention time (R_t) of 22.91, thus showing low polarity and was detected at 340 nm (Fig. 2).

Its UV spectrum (DAD) exhibited a broad band, with a maximum at 340 nm, associated with the cynnamoilic portion of the molecule; another one at 251 nm, relevant to the benzenic moiety and a third one around 304 nm, related to the pyronic group (Fig. 3). After different irradiation intervals, the changes appearing in chromatograms were analysed.

With increasing time of photoexposure, the area of the peak relative to the parent compound gradually decreased, while a larger number of photoproducts was progressively detectable.

As shown in Fig. 4, within 2 min from the beginning of irradiation, the chromatograms revealed the presence of four

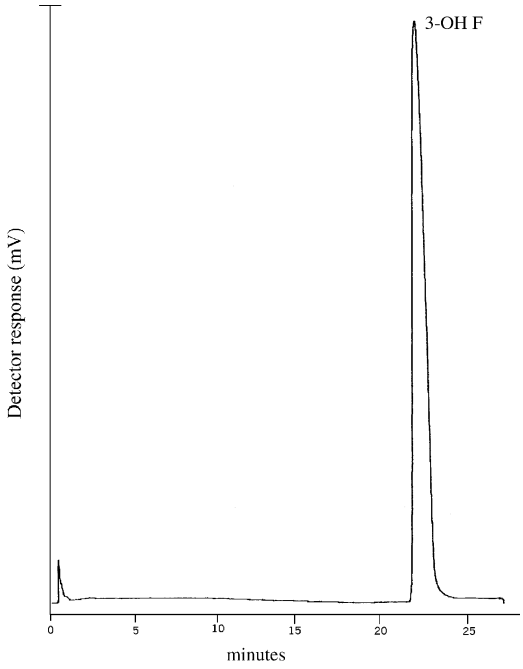

Fig. 2. Chromatogram of pure 3-OH-flavone before irradiation.

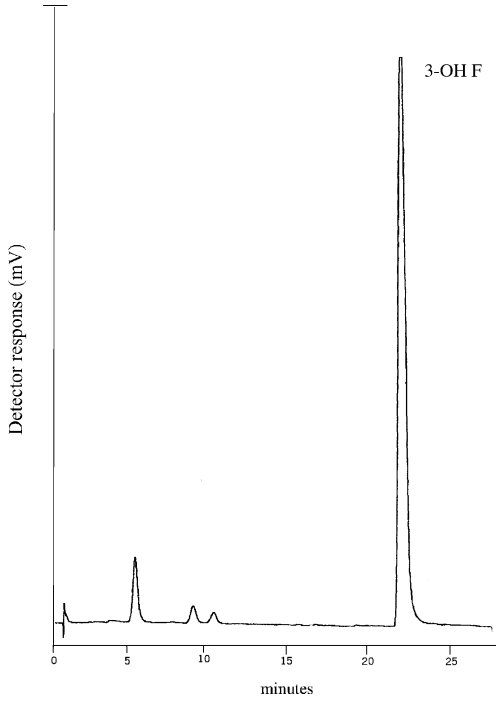

Fig. 4. Chromatogram of 3-OH-flavone exposed to 2 min of UV-A irradiation and its photoproducts.

photodegradation products, characterised by retention times of 4.12, 6.18, 9.92 and 11.25 min.

UV spectra, obtained with DAD system, of the corresponding HPLC peaks, showed the disappearance of the cynnamoilic band (340 nm), while the benzenic one (251 nm) was still evident for the species eluted at R_t = 4.12 and 6.18 min. The photoproducts with longer retention times (R_t = 9.92 and 11.25) exhibited only one new absorption band, centred around 280 and 276 nm, respectively (Fig. 5a–d). After longer irradiation times (3–11 min), nine additional new species were observed in the chromatograms, with retention times in the range from 2.46 to 20.07 min (Figs. 6 and 7), all absorbing in the spectral region between 246 and 259 nm. Retention times and UV-Vis absorption profiles of the new species obtained upon photolysis showed an increased polarity with respect to the reagent, consistently with the reaction pathway proposed.

Flavonols photochemistry in solution has been rationalised on the basis of a photo-rearrangement, mediated by intramolecular proton transfer in the excited state (ESIPT).

3.2. Validation of the HPLC method

The chromatographic method developed for the quantitative determination and separation of the photoproducts was validated according to ICH guidelines, to obtain

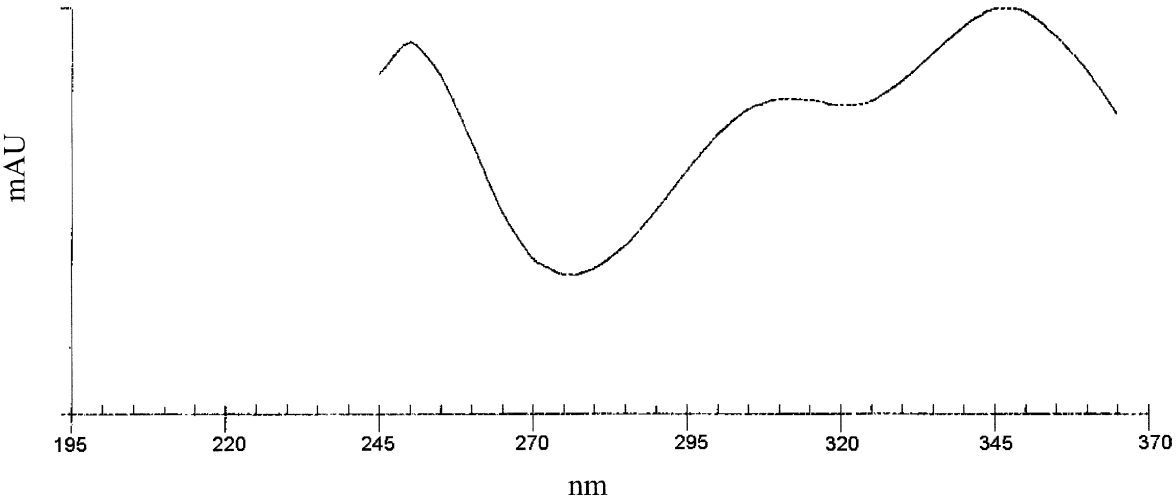

Fig. 3. UV-Vis spectra of 3-OH-flavone (R_t = 22.91).

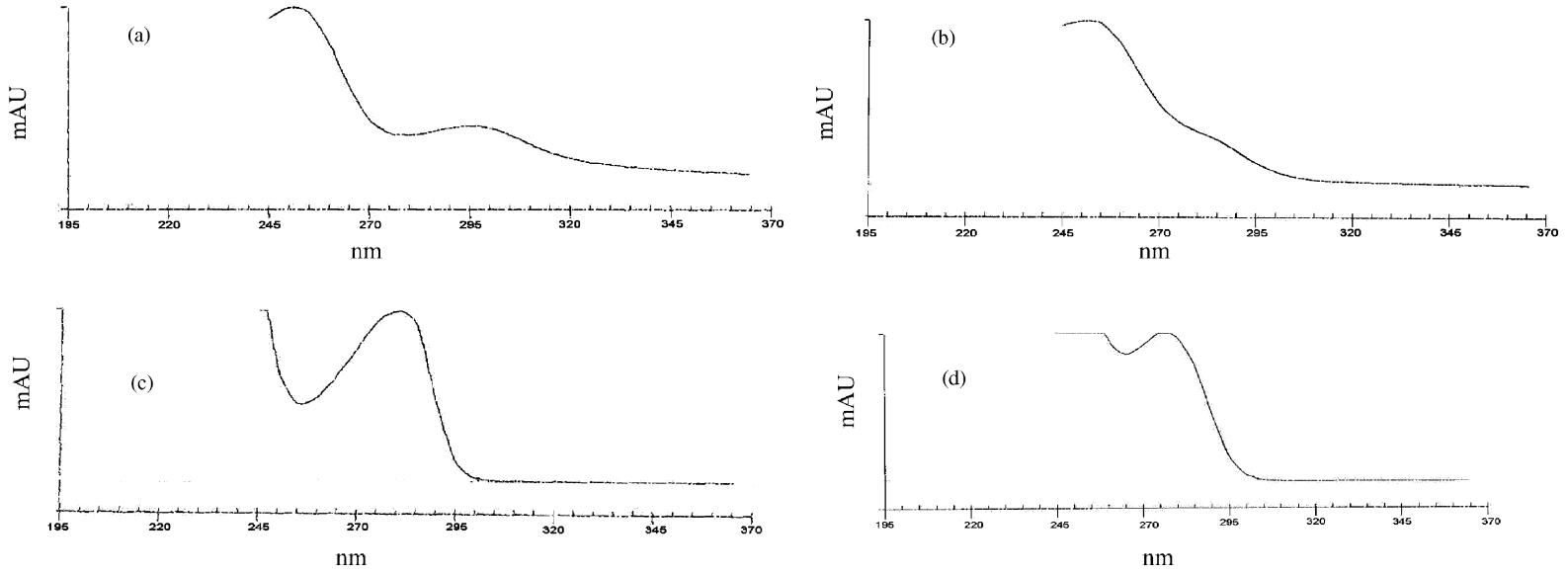

Fig. 5. UV-Vis spectra of (a) photoproduct eluted at $R_t = 4.12$; (b) photoproduct eluted at $R_t = 6.18$; (c) photoproduct eluted at $R_t = 9.92$; (d) photoproduct eluted at $R_t = 11.25$.

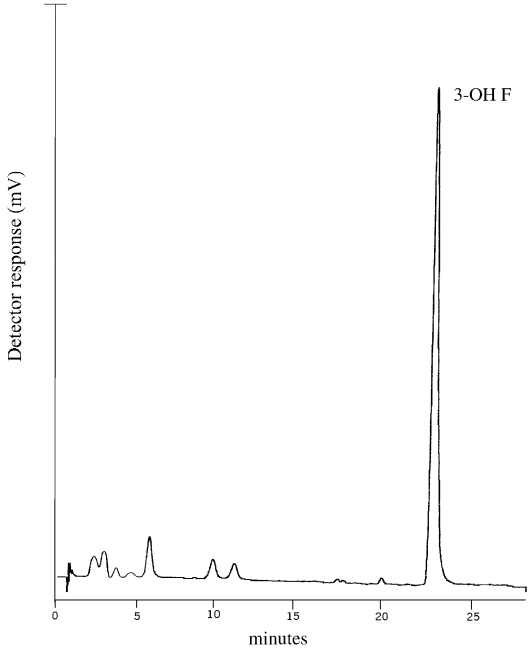

Fig. 6. Chromatogram of 3-OH-flavone exposed to 7 min of UV-A irradiation and its photoproducts.

reproducible analyses with a high degree of accuracy and precision in the range of concentrations investigated. The ICH parameters considered were specifity, linearity and range, precision and accuracy [16–19].

The optimum detection of 3-OH F was obtained at 340 nm. The HPLC protocol showed that this absorbance wavelength involves only the parent compound, thus allowing its quantitative evaluation without any interferences arising from the photoproducts, which all absorb in the spectral range between 246 and 280 nm.

Thus specificity derived from the quantitative analysis of the photodegradation of 3-OH F performed at the fixed wavelength of 340 nm. Furthermore, it was given by the high resolution factor ($R_s = 3$) between the peaks of 3-OH F and the nearest eluted compound ($R_t = 20.27$).

A calibration curve for 3-OH F was obtained from triplicate analyses at five concentration levels (1.45, 2.53, 5.06, 12.65, 25.3 μg/ml) and average values of the peak areas were subjected to linear regression analysis. Under the experimental conditions, a linear relationship was found between the peak area and the drug concentration ($r^2 = 0.997$; slope = 46577.08; intercept = −12592.4).

As regards sensitivity, the limit of quantification (LOQ) was calculated, according to ICH, from the standard deviation of the blank, referred to the slope of the calibration curve: LOQ = 1.45 μg/ml. The accuracy and precision of the assay were established across the specified range of the analytical procedure from intra- and inter-assay replicate analyses (Table 1). Each of the five concentration levels, covering the specified range, was analysed in triplicate. Accuracy was assessed as quantitative recovery percentage; the drug content was found to be between 79.07 and 131.17% of the claimed content, with an average value of 98.99%. For assessing repeatability, the mean and relative standard deviation (R.S.D.) were calculated at each concentration. R.S.D. for the intra-day assay was found to be between 1.18 and 4.20% (average R.S.D. = 2.46%); R.S.D. for the inter-day assay, calculated within two days, was found to be between 0.57 and 20.45% (average R.S.D. = 10.05%). The results showed that values obtained were in agreement with accepted validation procedure [20,21].

3.3. Kinetic model

The photodegradation kinetic of 3-OH F was evaluated from the HPLC corresponding peak areas at the selected irradiation times, by interpolation of the calibration curve previously established [22–24].

The degradation profile of the molecule showed a negative exponential plot of remaining drug concentration against time (s), according to an apparent first-order kinetic (Fig. 8),

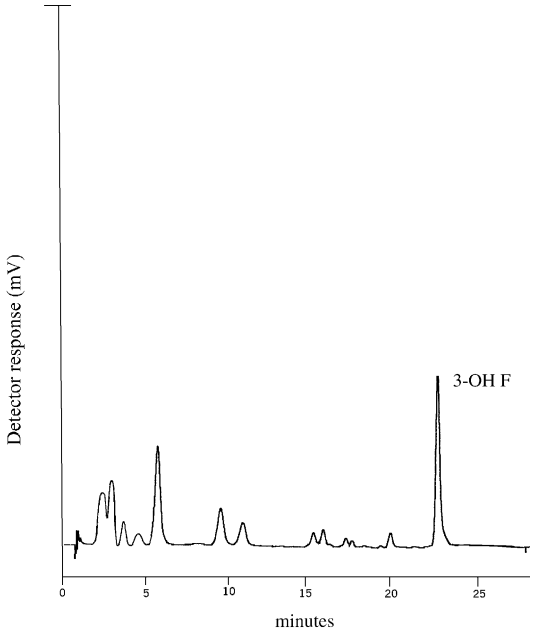

Fig. 7. Chromatogram of 3-OH-flavone exposed to 11 min of UV-A irradiation and its photoproducts.

Table 1
Accuracy, inter- and intra-day precision of the proposed HPLC method[a]

[3-OH-flavone] (μg/ml)	Recovery (%)	Intra-day R.S.D. (%)	Inter-day R.S.D.[b] (%)
1.45	98.82	1.72	20.45
2.53	102.18	4.20	11.15
5.06	79.07	1.18	15.14
12.65	83.70	2.58	2.94
25.3	131.17	2.60	0.57
Average	98.99	2.46	10.05

[a] Each data is the average of three determinations.
[b] Experiments were carried out within 2 days.

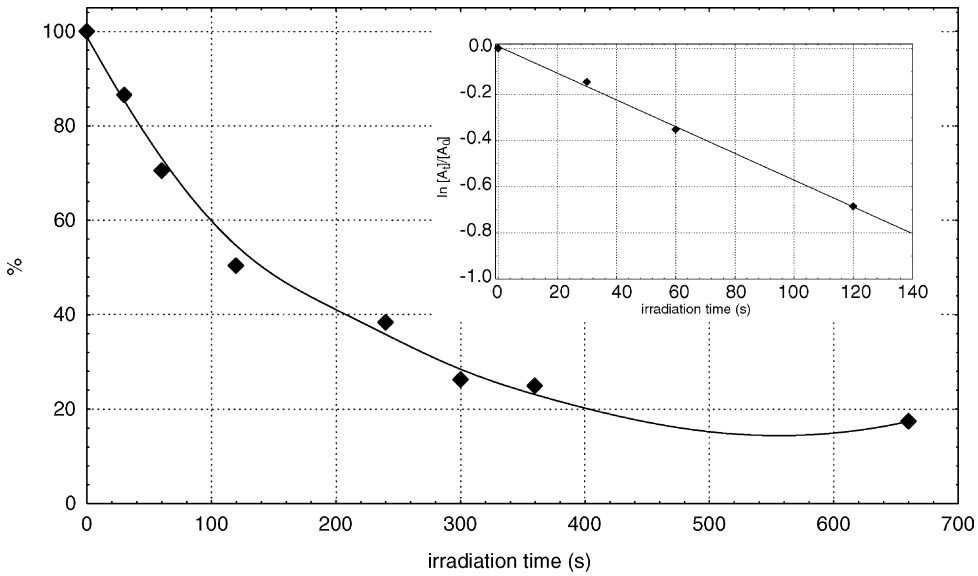

Fig. 8. Kinetic plot of light induced degradation of 3-OH-flavone.

according to the following equation:

$$\frac{A_t}{A_0} = e^{-k_a t} \quad (1)$$

where A_t and A_0 are the concentration of 3-OH F at time t and initially, respectively, k_a is the apparent first-order degradation rate constant.

This process was explained in more detail by estimating, from Eq. (1), the following parameters: degradation rate constant ($k = 6 \times 10^{-3}\,s^{-1}$); half-life time ($t_{0.5} = 1.55$ min); time of 10% decomposition ($t_{0.1} = 17.56$ s).

The degradation rate constant was calculated from the slope of the linear plot of ln $[A_t/A_0]$ versus time, as shown in Fig. 8 ($r^2 = 0.992$).

For longer irradiation times (3 min), the law order of the photochemical reaction can hardly be unequivocally assessed, probably due to a reaction mechanism of increasing complexity, involving contemporary and concurrent reactions.

4. Conclusions

The assay method proposed for the selective quantitation of 3-OH F proved to be suitable to obtain a good separation of photodegradation products from 3-OH F, thus overcoming any interferences on its determination.

The analytical method was validated in terms of specificity, limit of quantification, linearity and range, precision and accuracy.

The evaluation of the photochemical degradation allowed the estimation of kinetic parameters (k, $t_{0.5}$, $t_{0.1}$), really of great concern in pharmaceutics and requested by pharmacopoeias to predict stability during storage and handling.

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