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Photostability of drugs: photodegradation of melatonin and its determination in commercial formulations

V. Andrisano [a], C. Bertucci [b], A. Battaglia [c], V. Cavrini [a,*]

[a] *Dipartimento di Scienze Farmaceutiche, Universita di Bologna, Via Belmeloro 6, 40126 Bologna, Italy*
[b] *C.N.R., C.M.S.O.A., Dipartimento di Chimica e Chimica Industriale, Pisa, Italy*
[c] *Istituto C.N.R., I.Co.C.E.A., Via Gobetti 101, Bologna, Italy*

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Abstract

The photostability of melatonin, a hormone used as supplementary drug in the alleviation of jet-lag and other sleep disorders, was studied. The drug photodegradation at different pH values was monitored by HPLC methods. The main photoproduct was isolated and characterised on the basis of the NMR, FTIR, and mass spectra. A HPLC method, in combination with a post-column on-line photochemical derivatisation was developed for the selective and reliable quality control of commercially available melatonin containing products.

Keywords: Melatonin; Photostability; Liquid chromatography (HPLC); Photoproduct characterisation; Formulation quality control

1. Introduction

Many pharmaceutical compounds are known to be unstable to light [1–3] and have to be stored in sunlight-protected containers; moreover, significant phototoxic effects have been ascertained for several drugs. Accordingly, there is now an increasing attention to photochemistry of bioactive compounds including identification of their photodegradation products and control of their photobiological effects. All these aspects demand that selective, stability-indicating analytical methods are available.

Several classes of drugs (antimalarials, phenothiazines, non-steroidal antiinflammatory agents, etc.) have been deeply investigated for their photochemical and photobiological properties, whereas only poor information is available for other bioactive compounds.

The present communication deals with the study of the photoreactivity of melatonin, (*N*-acetyl-5-methoxytryptamine), a hormone produced mainly by the pineal gland from the aminoacid precursor L-tryptophan. This hormone acts as an internal clock for the timing of daily event and as such plays a crucial role in cueing circadian rhythms (sleep–wake cycle). At present indications for therapeutic applications of melatonin are circadian rhythm, sleep disorders, in-

* Corresponding author. Fax: +39-51-2099734.
E-mail address: vcavrini@alma.unibo.it (V. Cavrini)

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somnia in blind people, intercontinental flight disrhytmia (jet-lag syndrome) and insomnia in elderly patients [4,5].

The compound is reported to be photolabile, but detailed investigations on its photochemistry have not been performed.

The aim of this work was to assess the light stability of melatonin in solution and to define its photodegradation process. Moreover, the photoreactivity of the compound was exploited to develop a selective liquid chromatographic (HPLC) method, involving a post-column on-line photochemical reaction, able to confirm the identity of the compound in commercial formulations.

2. Experimental

2.1. Chemicals

Melatonin was obtained from Sigma (Milan, Italy), methanol and acetonitrile of HPLC grade were from Romil (Delchimica S.p.A, Milan, Italy); all other chemicals were from C. Erba Reagents (Milan, Italy). Purified water from a TKA ROS 300 System was used to prepare buffers and standard solutions.

Triethylammonium (TEA) acetate buffer (pH 7 and 8; 0.05 M) was prepared by adding acetic acid to 0.05 M triethylamine (TEA) aqueous solution up to desired pH value; 0.01M phosphate buffer solutions (pH 7.4; pH 4.0; pH 9.0) were prepared according to standard methods. Standard solutions of melatonin were prepared in methanol-0.01 M phosphate buffer 15:85 (v/v) at the desired pH value.

2.2. Apparatus

The HPLC system consisted of a HPLC 1050 Ti series pump, equipped with a Reodyne model 7125 injector with a 20 μl loop. The eluents were monitored by a Diode Array Detector (DAD) HP 1050 connected to a computer station. The detector wavelength was set at 225, 275 and 350 nm. The HPLC analyses were performed on a 5-μm C-18 Luna Phenomenex (150×2.0 mm i.d.) column, while semipreparative separation were carried out on a 10 μm Hypersil ciano-column (250×10 mm i.d.) using a 100 μl loop. The mobile phase composition (reversed phase and normal conditions) were adjusted according to the reported applications.

^{1}H NMR and ^{13}C NMR spectra were recorded on a 400 Mhz Mercury Varian spectrometer with Me4Si or $CHCl_3$ (in $CDCl_3$) as internal standards. Infrared spectra were recorded on a FT-IR Perkin Elmer Spectrum BX spectrometer. Mass spectra were recorded on a GCQ plus (Thermo Quest Finningan) mass spectrometer using a direct insertion probe (DIP) with the direct probe controller (DPC). The method allowed the sample to be directed introduced into the ion source.

Photochemical studies were carried out using a xenon arc source as artificial radiation system. In particular, a 150 W xenon-arc lamp (solar simulator, model 68805 Oriel corporation, USA) was used, provided with a dicroic mirror to block visible and IR radiation to minimise the sample heating. An air-mass filter (mod 81090) was also used to simulate solar conditions. The output beam was directed downward by a 'beam turning assembly', which contain the dicroic mirror. The UV dose (J cm^{-2}) from the Xe-arc lamp was measured by a radiometer (Goldlux, mod 70127, Oriel Corporation, USA).

Post-column photochemical derivatization was performed using a Beam-Boost photochemical reactor (ICT, Frankfurt, Germany) arranged on-line between the analytical column and the photodiode array detector. The elute was irradiated on-line in capillary PTFE tubing (10 m 0.3 mm i.d.) in crocheted geometry by a 8-W-low pressure mercury lamp with prominent spectral emission at 254 nm.

2.3. Photostability testing

2.3.1. Preliminary tests in flow

A system consisting of HPLC pump, Reodyne injection valve, photochemical reactor and diode array detector was used without analytical column. A solution of melatonin (0.056 mg ml^{-1}) in methanol–pH 7.4 phosphate buffer (0.1 M) was then injected in a flow of methanol–pH 7.4 phosphate buffer (0.1M) 30:70 (v/v) at 0.3 ml

min^{-1}. The UV spectra of melatonin were recorded on-line with the UV-254 lamp of the photoreactor switched off and on and compared.

2.3.2. Exposure to solar simulator

Solutions of melatonin (0.14 mg ml^{-1}) in methanol–pH 7.4 phosphate buffer (0.01 M) 15:85 (v/v) into 3.5 ml-quartz cells were exposed to UV-A and UV-B radiation (Xenon arc-lamp) for prefixed times and monitored by HPLC to evaluate the compound photodegradation.

The experiments were repeated on pH 4.0 and pH 9.0 phosphate buffer solutions of the drug.

2.3.3. Sunlight exposure

Melatonin solution (0.22 mg ml^{-1}) in methanol–pH 9 phosphate buffer (0.01M) 15:85 (v/v) into a Pyrex volumetric flask was exposed to sunlight for three months and then analysed by HPLC on 5 μm C-18 Luna Phenomenex (150 × 2.0 i.d.) column using acetonitrile–pH 8 TEA acetate (0.05 M) 16:84 (v/v) at a flow rate of 0.3 ml min^{-1}.

2.4. Isolation of the photoproducts

Solution of melatonin (about 0.22 mg ml^{-1}) in methanol–pH 9 buffer solution (0.01 M) 15:85 (v/v) were exposed to UVA and UV-B radiation (solar simulator) or to sunlight. Then the irradiated solution was extracted with an identical volume of ethylacetate-n-hexane 1:1 (v/v) into a separator; the organic phase was removed, the aqueous phase was saturated with sodium chloride and extracted with ethyl acetate. The organic solvent was removed under reduced pressure and the residue was dissolved with 1 ml of methanol. The resulting solution was subjected to semipreparative chromatographic separation on a CN-column (250 × 10 mm i.d.) using n-hexane-2 propanol 85:15 (v/v) at a flow rate of 2.5 ml min^{-1}. with UV detection at 270 nm. The eluate fraction corresponding to the main photodegradation product of melatonin was collected and evaporated to dryness. The residue was used for subsequent NMR, Mass spectrometry and FTIR analyses.

^{1}H NMR ($CDCl_3$) δ 1.99 (s, 3 H), 3.3 (t, 2 H), 3.66 (q, 2 H), 3.85 (s, 3 H), 6.05–6.15 (b, 1 H), 7.16 (m, 1 H, $J_1 = 9.0$ Hz, $J_2 = 3.0$ Hz), 7.39 (d, 1 H, $J = 3.0$ Hz), 8.46 (d, 1 H, $J = 2.0$ Hz), 8.69 (d, 1 H, $J = 9.0$ Hz), 11.1–11.2 (b, 1 H);

^{13}C NMR ($CDCl_3$) δ 23.5, 34.6, 39.7, 55.8, 115.5, 120.8, 122.5, 123.1, 133.2, 154.7, 159.2, 170.0, 203.1.

IR ($CDCl_3$) cm^{-1}: 3600–3200, 1800, 1705, 1684.

MS spectrum: 264 (M^+;12); 203 (17); 192 (35); 176 (100).

2.5. Analysis of commercial melatonin tablets

2.5.1. Calibration graph

Standard solutions of melatonin (1–20 μg ml^{-1}) in water-methanol 80:20 (v/v) were subjected to HPLC analysis on 5 μm C-18 Luna Phenomenex (150 × 2 mm i.d.) using acetonitrile–pH 8 TEA acetate buffer (0.05 M) 16:84 (v/v) at a flow rate of 0.3 ml min^{-1}. The peak area were plotted against the corresponding melatonin concentration to obtain the calibration graph.

2.5.2. Assay procedure

An aliquot of powdered sample, equivalent to 2 mg of melatonin, was treated with water-methanol 80:20 (v/v), the resulting suspension was filtered and the clear solution was diluted to 50 ml with the same solvent. After further dilution (1:5) the solution was filtered through a 0.45 μm Millipore filter and then subjected to HPLC analysis as reported under calibration graph. The melatonin content was calculated by comparison with an appropriate standard solution (8 μg ml^{-1}).

3. Results and discussion

The present study was focused on the photochemical reactivity of melatonin and on its selective determination in commercial preparations.

3.1. Photochemistry

Preliminary information on the photoreactivity of melatonin was obtained by using a photoreac-

tor arranged in a HPLC system without the analytical column. The analyte, injected in a flow of methanol-pH 7.4 buffer solution 30: 70 (v/v), is subjected to UV-254 radiation as it moves (0.3 ml min^{-1}) into a capillary to the Diode Array Detector (DAD); light-induced modifications of the chromophore result in marked alterations of the UV spectra. In Fig. 1 the comparison of the melatonin UV spectra obtained with photoreactor lamp Off and On suggests that significant modifications in the melatonin chromophore occurred. These data obtained with this simple and rapid (about 5 min) procedure was then confirmed by studies on melatonin solutions exposed to UV-A (320–400 nm) and UV-B (290–320 nm) radiation (Solar simulator) and to sunlight.

Accordingly, photodegradation of melatonin was evaluated in buffer solution at pH 7.4 (physiological value), pH 4.0 and 9.0 containing 15% of methanol to ensure adequate solubility. The concentration of melatonin and its photoproducts was monitored by RP-HPLC method; a representative HPLC chromatogram obtained from a pH

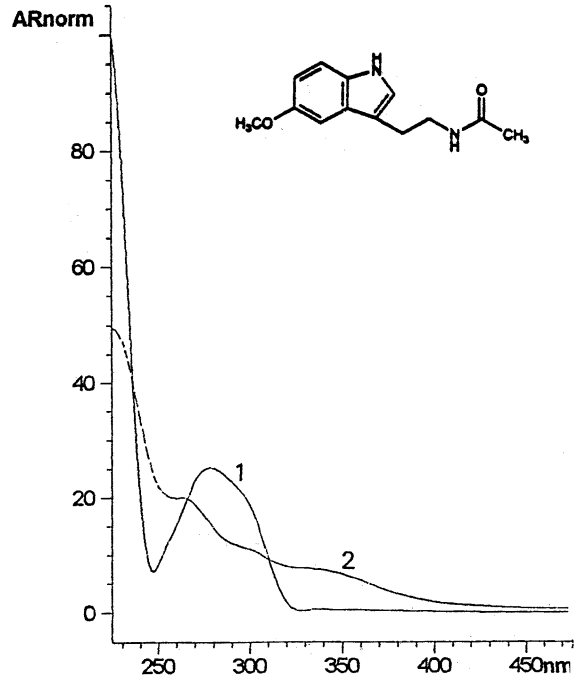


Fig. 1. Comparison of the melatonin UV spectra obtained with photoreactor lamp Off (1) and On (2).

7.4 melatonin solution exposed to the solar simulator for 1 h is shown in Fig. 2. As shown, a photoproduct (Rt = 9.66) more hydrophilic than melatonin (Rt = 14.2) is obtained as the main photodegradation product; its UV spectrum (Diode array detector) exhibits an absorption maximum at 345 nm (Fig. 3), according to the preliminary flow data (Fig. 1).

A series of photodegradation experiments were then carried out and the obtained results can be summarised as follows: (a) the photodegradation of melatonin is rapid at pH 9.0 and decreases as the solution pH decreases; (b) the same main photoproduct is obtained at the different pH values; (c) the photodegradation of melatonin is prevented in deaerated solutions, photoproduct being obtained only in the presence of oxygen; (d) light-protected melatonin solutions were found to be stable.

The photodegradation profiles of melatonin under the different pH condition are illustrated in Fig. 4. After long times light exposure a secondary product was obtained in low extent; at pH 9.0 the complete photodegradation of melatonin was obtained after exposure to UV-A and UV-B radiation for 17 h. Melatonin solution (pH 9.0) in pyrex volumetric flasks (UVBradiation protected) exposed to sunlight yielded the same main photoproduct at lower rate.

The rate of photodegradation of melatonin was evaluated in low concentration solutions. Under these conditions linear relationships were obtained according to the following equation: $\ln A = \ln A_0 - kt$ (apparent first-order kinetics), where A is the remaining peak area and t = time (min). The obtained data were: pH 9.0 (slope: -0.00730 ± 0.00077; $r^2 = 0.987$), pH 7.4 (slope: -0.00430 ± 0.00036; $r^2 = 0.960$), pH 4.0 (slope: -0.00197 ± 0.00007; $r^2 = 0.994$).

3.2. Elucidation of the photoproduct structure

A solution of melatonin (pH 9) was exposed to solar simulator or sunlight in order to obtain high yield of its main photoproduct. The irradiated solution was first extracted with n-hexane/ethylacetate 1/1 to remove the residual melatonin and then the aqueous phase was saturated with

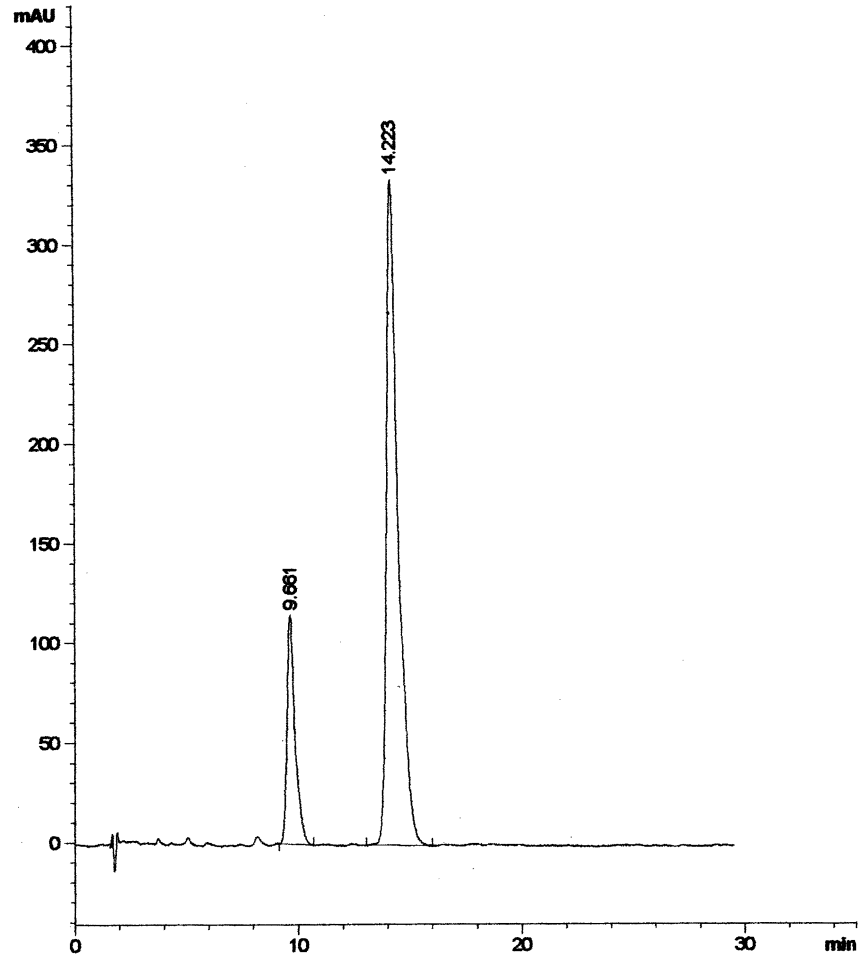


Fig. 2. Representative HPLC chromatogram obtained from a pH 7.4 melatonin solution exposed to the solar simulator for 1 h: photoproduct (Rt = 9.66), melatonin (Rt = 14.2). Chromatographic conditions: 5 μm C-18 Luna Phenomenex (150 × 2.0 i.d.) column, methanol–pH 7 TEA acetate (0.05 M) 30:70 (v/v) at a flow rate of 0.3 ml/min.

sodium chloride. Finally the photoproduct was provided by ethylacetate extraction. A subsequent chromatographic purification on ciano column allowed to obtain a suitable product for structural characterisation. In particular, mass spectrum gave 264 as the molecular peak ion, this suggesting the addition of an oxygen molecule to melatonin (M 232). Indeed the presence of oxygen was proved to be essential for the melatonin photodegradation.

The structure was then elucidated by ^{1}H, ^{13}C NMR spectroscopy, while attached proton test (APT) and distorsionless enhancement by polarisation transfer (DEPT) experiments were performed to distinguish the different carbon atoms. In particular, the resonance doublet at 8.46 ppm (HC=O) collapsed into a singlet after irradiation of the broad signal at 11.15 ppm (NH). The aromatic system featured only three resonances, namely at 7.16 and 8.69 ppm for the two vicinal hydrogens ($J = 9.0$ Hz), and 7.39 for the meta hydrogen ($J = 3.0$ Hz). The ^{13}C NMR showed prominent resonances at 203.1 ppm for the C=O substituent, and at 170.0 and 159.2 ppm for the carbon atoms of the formamido and acetamido substituents, respectively.

Finally IR showed a broad band in the 3600–3200 cm^{-1} region (NH) and absortions around 1700 cm^{-1}, thus confirming the presence of additional carbonyl groups with respect to the starting compound. Therefore the structure 3 (Scheme 1) was assigned to the main photoproduct of melatonin, 1. A possible mechanism for the photodegradation involves the photooxydation of the indole ring (Scheme 1) giving the endoperoxide 2 as a plausible reaction intermediate, which rapidly photorearranges to the stable product 3 [6].

3.3. Analysis of commercial formulations

Various analytical methods such as GC-MS [7] and HPLC with fluorescence [8,9] or electrochemical [10] detection have been reported for melatonin assay in biological samples, but few studies, involving GC-MS [11], HPLC-MS-MS [12] and TLC-densitometry [13] have been made for the determination of the compound in formulations. Melatonin containing products are now commercially available and used for the treatment of jet lag and sleep disorders. Therefore, there is need for simple and reliable methods for the quality control of these products. The reported GC-MS method [11] involve a chemical derivatisation step and the HPLC–MS-MS requires an expensive instrument. In order to provide a selective and practical method suitable for a common quality control laboratory, HPLC in combination with a post-column on-line photochemical reactor, exploiting the photoreactivity of melatonin was developed.

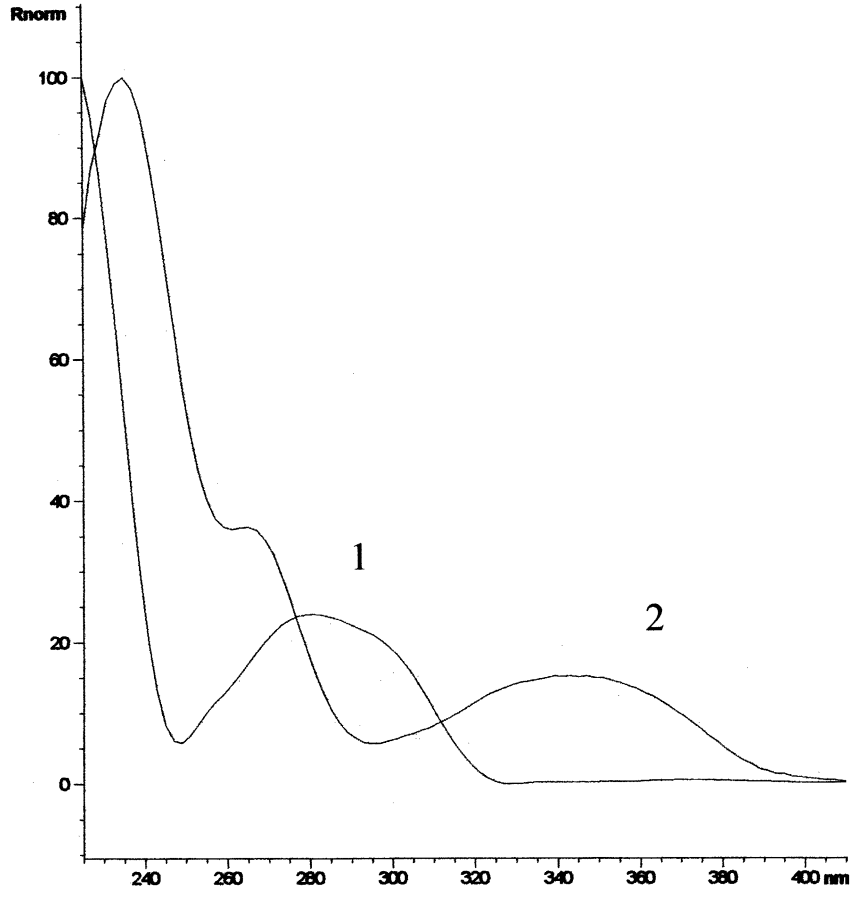
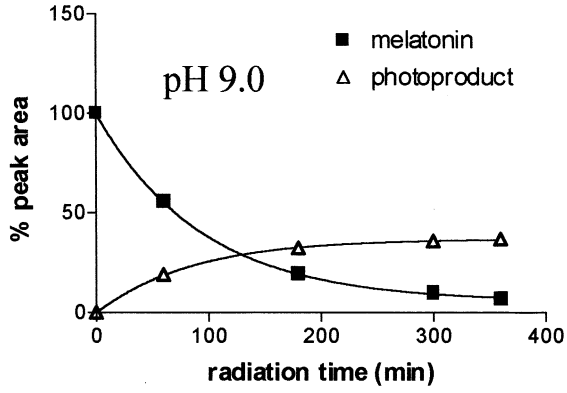
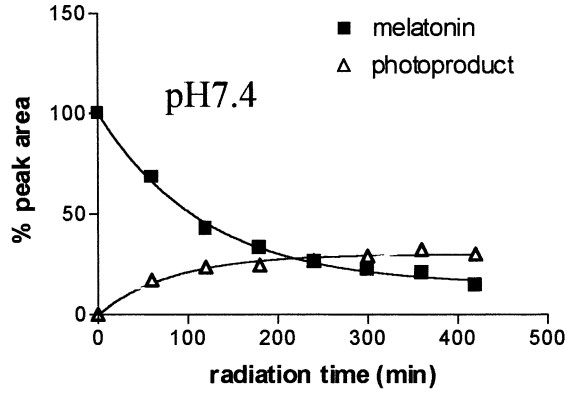


Fig. 3. UV spectra of (1) melatonin; (2) photoproduct recorded on peak apex with the DAD.





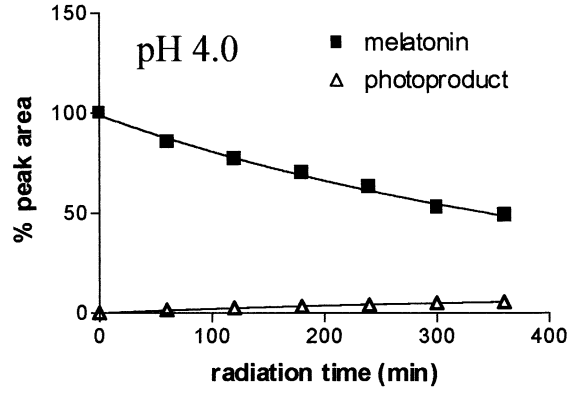


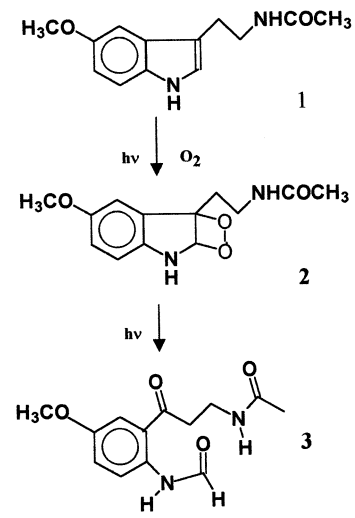
Fig. 4. The photodegradation profiles of melatonin under the different pH conditions

Flowing out of the analytical column, the analyte is subjected to UV radiation (254 nm) as it moves into the capillary around the lamp to the Diode Array Detector [14,15]. Light induced modifications in the drug chromophore resulted in significant spectral modification; therefore, two UV spectra (photoreactor lamp off and on) can be acquired for melatonin allowing its unambiguous identification. A representative chromatogram obtained from a commercial sample (Somnium, Kos) is shown in Fig. 5. The peak at Rt = 19 min is due to melatonin, whose identity was confirmed on the basis of its UV spectrum. In fact both the UV spectra (Photoreactor on and off Fig. 1) of melatonin from commercial samples were superimposable to the corresponding spectra from standard melatonin.

Using the same approach, also the chromatographic peak at Rt = 4.9 (Fig. 5) was identified as Vitamin B6.

Thus, this combination of spectrophotometric data with chromatographic parameters enhanced the intrinsic selectivity of the HPLC method.

For quantitative applications, a linear relationship between melatonin peak area and concentration (1–20 μg ml^{-1}) was verified ($r = 0.9972$; $n = 5$). The drug content was found to be 103.6% of the claimed content (RSD% = 0.84) ($n = 5$), and no detectable traces of photodegradation products were observed. The method accuracy was evaluated by recovery studies. Known quanti-



Scheme 1.

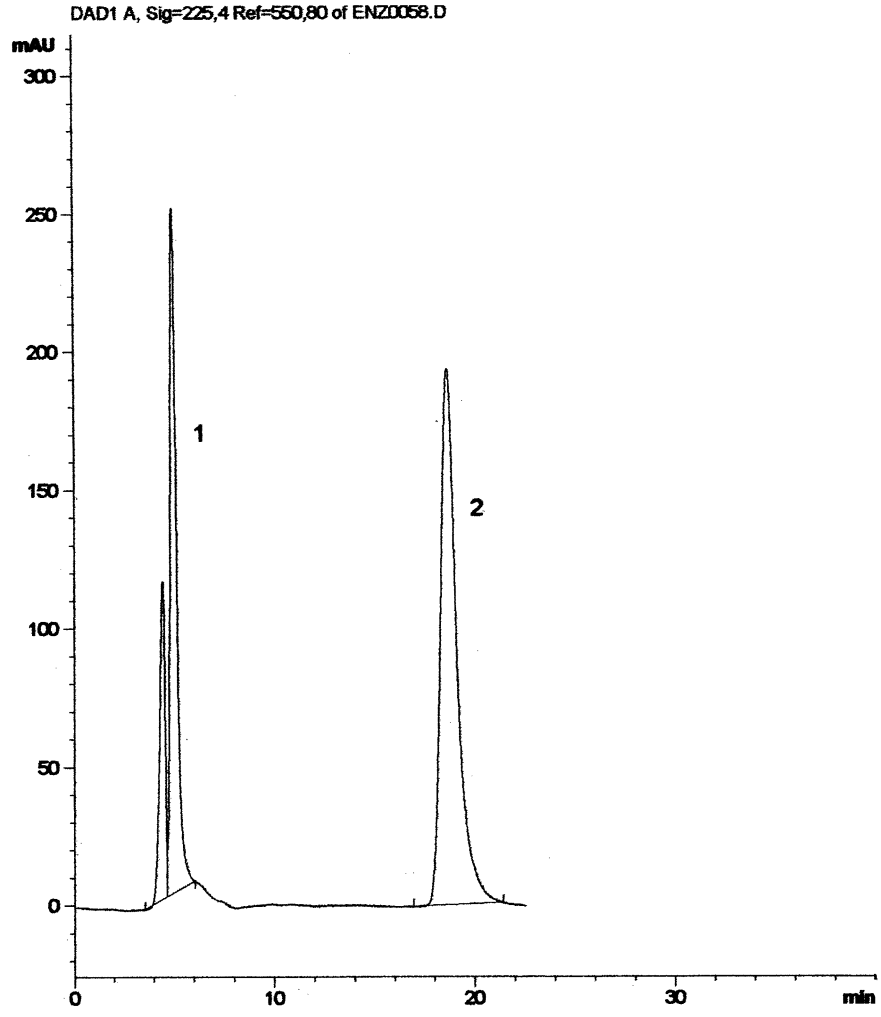


Fig. 5. Representative chromatogram from the analysis of a commercial sample: (1) piridoxine; (2) melatonin. Chromatographic conditions: 5 μm C-18 Luna Phenomenex (150 × 2.0 i.d.) column, acetonitrile–pH 8 TEA acetate (0.05 M) 16:84 (v/v) at a flow rate of 0.3 ml/min.

ties of melatonin were added to the powdered sample to obtain fortified samples (130 and 150%); quantitative recovery (98.7–99.5%) were obtained with good intraday precision (RSD% = 0.93; $n = 5$). Analyses ($n = 8$) for a commercial sample by two different analysts provided a mean value of 102.3% (RSD = 1.85).

4. Conclusions

The present study on the photoreactivity of melatonin, allowed to understand the mechanism of its photodegradation and to elucidate the structure of its main photoproduct. Moreover, the compound photoreactivity can offer the basis for a selective HPLC method suitable for a reliable quality control of commercial formulations.

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