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Short communication

Spectrofluorimetric assay for the photodegradation products of alprazolam

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Abstract

A new spectrofluorimetric assay for the photodegradation products of the ansiolytic drug alprazolam is described. Alprazolam was found to be highly photolabile and special care should be taken to avoid light exposure during alprazolam storage and handling. The photostability of alprazolam was evaluated at pH 2.0, 3.6 and 5.0. The drug was exposed to UVA–UVB radiations, the photodegradation of alprazolam was followed by high-performance liquid chromatographic (HPLC) and the developed spectrofluorimetric assay allowed determination of the photodegradation products at very low concentrations ($\geq 10^{-5}$ M). The photoinstability was found to increase with the pH value decreasing, consequently acidic media should be avoided during the drug-development process.

Keywords: Alprazolam quality control; Alprazolam photostability; Spectrofluorimetric assay; Benzodiazepine phototoxicity; Alprazolam stability

1. Introduction

Drug photostability constitutes an important current subject of investigation because the photodegradation process can result in a loss of the potency of the drug and also in adverse effects due to the formation of minor toxic degradation products [1–3]. Photochemical degradation of drugs and drug formulations has thus recently developed into an important field of research; this can partly be ascribed to the demand for harmonized guidelines for photochemical stability studies of drugs and drug products from regulatory bodies dealing with drug registration worldwide [4–6].

Alprazolam is one of the 1,4-benzodiazepinones that exhibit high milligram potency, and, therefore, therapeutic doses are low and concentration in plasma is correspondingly low [7]. Hydrolysis of the bendiazepinone ring is one the most frequently observed degradation routes for benzodiazepinones [8–10]; nevertheless, in the case of alprazolam, we have determined that hydrolysis under several different conditions, is not a major

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degradation source [11,12], similar observations have been reported for this [13,14] and other related triazole benzodiazepinones [15,16]. A reversible 1,4-benzodiazepinone ring-opening under aqueous acidic conditions have been early described for alprazolam [17] and for triazolam [18].

On the other hand, to the best of our knowledge, there are no reports on photochemical studies carried out with alprazolam, previous to our work. In preliminary accelerated aging studies we have observed that the drug is photolabile, but the UV-visible spectrophotometric method [19], and the high-performance liquid chromatographic (HPLC) method [20], usually used for the alprazolam assays, do not detect the main photodegradation products. More sophisticated methods such as HPLC–tandem mass spectrometry (electrospray ionization MS–MS), recently developed for the quantitation of alprazolam in human fluids [21–23] and hair [24], are not suitable to follow the drug degradation. The lack of sensitive methods to test the alprazolam photostability focused our attention on the development of a suitable procedure that it is described in the present communication.

2. Experimental

2.1. Materials

Alprazolam, some potential degradation products and related compounds were obtained from Gador Lab. and used as received. HPLC grade acetonitrile, citric acid, disodium acid phosphate and triethylamine from Aldrich were used as purchased. LC grade methanol was distilled immediately prior to use. Phosphate–citrate buffer solutions (pH 2.0, 3.6 y 5.0) were prepared according to standard methods.

2.2. Apparatus

The fluorescence analyses were performed using a Perkin Elmer, LS-5 Luminescence Spectrometer. The UV spectrum was recorded using a Shimadzu, UV-1601PC spectrophotometer. The HPLC analyses were performed using a quaternary HP G1311A series pump, equipped with a Rheodyne Model injector with a 20 μl sample loop. The eluates were monitored by a multiwavelength UV detector HP 61315A connected to a computer station (HP Chemstation, G2170AA); for routine analyses the wavelength was set at 220 nm. The chromatographic separations were performed on a reverse-phase RP-18 Lichrosorb, 5 μm, (200 × 4.6 mm) column, and the mobile phase composition was adjusted according to the reported applications. Sodium phosphate buffer (pH 3.5, 0.05 M) (A)–acetonitrile (B) under isocratic conditions (A:B = 35:65) (buffer: 5×10^{-2} M phosphoric acid, 1.44×10^{-2} M triethylamine, adjusted to pH 3.5 with sodium hydroxide). The flow rate was 1 ml min^{-1}. Aliquots of the irradiated samples were extracted with methylene chloride, dried with $MgSO_4$ and the solvent distilled at reduced pressure; each extract was diluted in the mobile phase to a 10^{-4} M final concentration and 20 μl aliquots were injected.

2.3. Photostability studies

For the UV radiation exposure testing a photoreactor provided with a Medium-Pressure Metal Halide Lamp HPA-400 W (Phillips) and mirrors was used. Distance between the lamp and the samples was 5 cm; likely heating of the samples was monitored and the temperature was always below 60 °C. The UV dose from the lamp was measured by a reported method [25,26] using quinine monohydrochloride dihydrate (two solutions in water) as a chemical actinometer, for calibrating the UV radiation in sunlight simulating conditions. The calibration was carried out following the recommendations of the International Conference on Harmonization (ICH), the unit of integrated intensity of radiation is expressed as the difference in absorbance, ΔAbs, at 400 nm of the irradiated quinine solution [27]. Under the present conditions, the integrated intensity of radiation after 6 h exposure was of 0.9 quinine units.

2.4. Assay procedure

Alprazolam solutions (3×10^{-3} M) prepared in pH 2.0, 3.6 and 5.0 buffers into 50 ml volumetric flasks tightly capped with teflon caps, were irradiated for at least four half-lives in each case. Preliminary assays were carried out to determine the half-lives by measuring the residual Alprazolam concentration by HPLC at several time intervals, in samples irradiated under the same conditions that those described for the spectrofluorimetric determinations. Five milliliters of aliquots of the irradiated solutions were periodically removed and analyzed immediately. The photodecomposition was determined by measuring the emission at 435 nm after exciting with a 260 nm wavelength. The kinetics was followed for at least four half-lives. In all cases the fluorescence of a non-irradiated Alprazolam solution under the same conditions was measured as a blank, the observed value was always nul. The residual Alprazolam concentration at the final time was measured by HPLC, i.e. after 120 h for the runs at pH 5.0. Similarly, buffer solutions (no containing alprazolam) were irradiated under the same conditions for 120 h, and no fluorescence was detected.

3. Results and discussion

Acceleration tests on the photochemical reactivity of alprazolam were carried out using a Medium-Pressure Metal Halide Lamp as an artificial radiation system for simulating natural sunlight exposure. The UV spectrum of alprazolam exhibits two maxima at 240 and 260 nm and, therefore, the UV components of the sunlight can be considered the main responsible for the photochemical reactivity of the drug. The controlled conditions described hereby constitute a viable option according to the ICH [2,5] guidelines and are very similar to other recently reported photodegradation studies [1,16]. A yellowish color developed after 15 days exposure of the alprazolam buffered solutions at daylight, and the color increased progressively along the exposure time. Similar changes were observed when buffered solutions were irradiated with a UV light. The UV-visible spectrum of the solutions show a decrease in the absorbance of the band at λ_{max} 260 nm and a simultaneous increase in the absorbance of the band at λ_{max} 240 nm, but the changes, even after relatively long irradiation times, are not enough for a quantitative determination of the drug photostability. Since the UV spectra of the non-irradiated solutions do not change along the time under the present reaction conditions, the observed changes in the UV spectra of the irradiated solutions are not associated with hydrolysis in acid medium as previously described [19].

The HPLC method frequently used for the quantitative determination of alprazolam is not suitable to determine the photodegradation products. As an example, when 1.0 ml aliquots of a 2.92×10^{-3}M solution of alprazolam at pH 3.6 (buffer: 2×10^{-4} M citric acid 2×10^{-4} M Na_2HPO_4) in sealed ampoules were irradiated at room temperature, complete degradation of alprazolam was observed after 25 h. (Fig. 1(A) and (B)) shows the first order decay in the first 8 h, a half-life time of 4.8 h was determined, but not new signals appeared in the chromatogram. Several different HPLC conditions were tested to detect the main photodegradation products but they were unsuccessful.

Similar photodegradation studies were then carried out at three different pH, and the samples analyzed applying the spectrofuorometric assay described in the Section 2, by measuring the increase in the fluorescence signal at 435 nm. The content of remaining alprazolam was simultaneously determined by HPLC. Control assays carried out in the dark showed no fluorescence throughout all the exposure time and no changes in the alprazolam content.

3.1. Photodegradation kinetics

Following recommendations for preliminary photodegradation studies [1,25,26,28], the kinetics of the alprazolam photodegradation was determined in low concentration buffered aqueous solutions, so that the drug does not absorb all the light available and the reaction rate is limited by the drug concentration. Buffered aqueous solutions of the drug at pH 2.0; 3.6 and 5.0 were

exposed to UVA–UVB radiation and monitored by determining their fluorescence spectra by the procedure described hereby. At the three pH's the kinetics of the photodegradation follows a first-order law described by Eq. (1) where F and F_0 are the fluorescence at 436 nm, ($\lambda_{exc} = 260$ nm), at time t and $t = 0$, respectively.

$$\ln\left(\frac{F}{F_0}\right) = kt \quad (1)$$

Fig. 2 shows the resulting profiles for the formation of the photodegradation products at the three pHs. As shown, linear plots of $\ln(F/F_0)$ against time (min) were obtained at pH 2.0 (correlation coefficient, $r = 0.98$); pH 3.6 ($r = 0.98$); pH 5.0 ($r = 0.97$), according to apparent first-order kinetics. It can be observed that the photodegradation increases as the pH value decreases. The regression analysis was carried out by applying the least-squares method and the following kinetic parameters were obtained: rate constants k: 3.61×10^{-5} s^{-1} (pH 2.0); 1.67×10^{-5} s^{-1} (pH 3.6) and 5.55×10^{-6} s^{-1} (pH 5.0); and half-life times, $t_{0.5}$ 5.3 h (pH 2.0); 11.5 h (pH 3.6) and 34.7 h (pH 5.0). These kinetic parameters are dependent of experimental conditions (radiation source, sample irradiation geometry, ionic strength, solvent, etc), nevertheless, their relative values are of practical utility suggesting the need for appropriate light protection of alprazolam for its handling and storage, and specially during the pharmaceuticals production. They also show the particular photolability in acidic media.

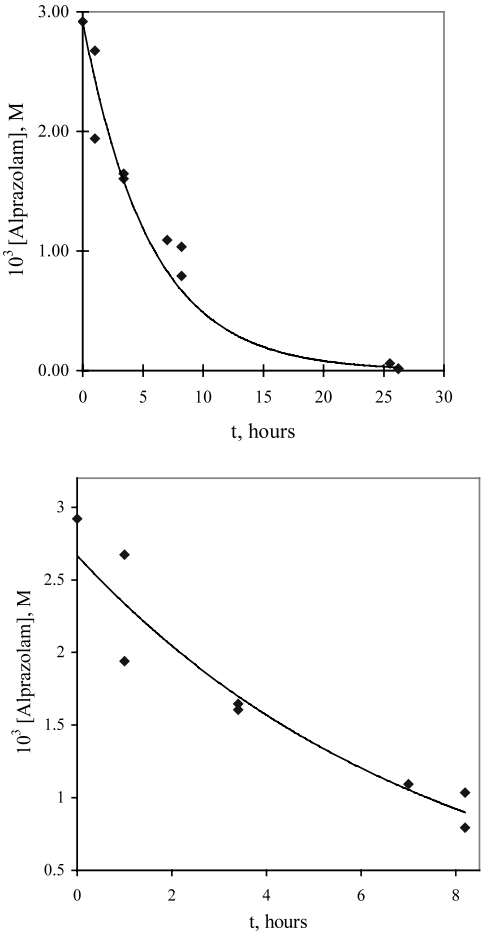


Fig. 1. Photodegradation of Alprazolam. HPLC determinations of buffered solutions (pH 3.6) irradiated for: A) 25 h; B) 8 h.

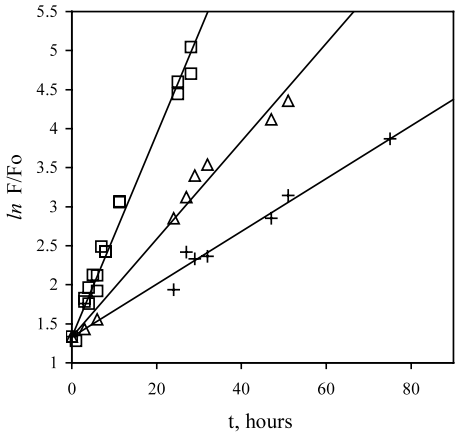
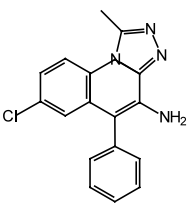


Fig. 2. Formation of the photodegradation products of Alprazolam followed by fluorescence determinations [□] pH 2.0; [Δ] pH 3.6; [+] pH 5.0.

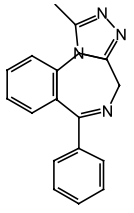
3.2. Photodegradation products

It has been recently reported that antidepressants may cause photosensitivy [27,28], [29,30], and some cases of photosensitivity reactions due to alprazolam has been recently reported in patients taking from 0.25–1.2 mg daily dosis [31–33]. It was then of special interest trying to isolate and characterize the structures of the main

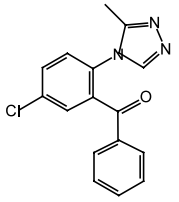
photodegradation products, in relation to their potential phototoxicity. Photodegradation for this purpose was carried out at pH 2.0 using 1 l of a buffer phosphate citrate solution. One gram of Alprazolam was first dissolved in 130 ml of methanol and added to the buffer solution, to get a final concentration of 3.2×10^{-3} M. Aliquots were irradiated for 10 days. Several attempts should be carried out to produce the main photoproducts and, since the structures are very similar, many different systems were assayed to isolate each of them [34]. The structures of the three main photoproducts **II**–**IV** were elucidated by ^{1}H and ^{13}C NMR spectroscopy and HRMS, and confirmed by independent synthesis [1], they are fully stable under the conditions described in this paper.



II



III



IV

The main photodegradation product is the triazolaminoquinoleine, **II**, which exhibits a strong fluorescence as well as compound **III**, while the 5-Chloro-[5″-methyl-4H-1,2,4-triazol-4-yl]benzophenone (**IV**), shows no fluorescence. Compounds **II**, arises from photochemically catalyzed rearrangements of the seven-membered ring to a six-membered ring, the extended delocalization in these systems may be the responsible for the observed high fluorescence. Compound **III** is formed by the UV-promoted dechlorination of alprazolam [34]. Future in vitro studies of the possible phototoxicity of these two compounds, are planned.

On the other hand, compound **IV**, which is produced in low amounts, arises from the opening of the benzodiazepinone ring, a phenomenum usually observed in major extent in other benzodiazepinone drugs [9,10,35]. Isolation and characterization of other two photodegradation products, which appear only in very low concentration, are currently in progress.

3.3. Validation

Validation of the method was carried out by preparing sets of samples A and B, with the photo degradation products that were found to be fluorescent, products **II** and **III**, respectively. Compounds **II** and **III** were independently synthesized [34] and sets of samples ($n = 6$), containing concentrations in the range $= 0.4–1.3\ 10^{-3}$ M for **II** (set A), and $0.3–0.9\ 10^{-3}$ M for **III** (set B), were prepared. Regression analysis of the calibration graphs demonstrated linearity with slopes 56.88 ± 2.72 ($r = 0.990$) and 62.47 ± 1.87 ($r = 0.994$), for **II** and **III**, respectively, in the concentration ranges given.

The accuracy and precision of the assay near the limit of quantitation (LOQ) and two addi-

Table 1
Inter- and intra-assay precision (RSD) and accuracy for alprazolam photodegraded products **II** and **III** (see the text)

Photodegradation product	Concentration 10^3 M	RSD		Recovery%
		within-day	between-day	
Triazoloamino-quinoleine (**II**)	0.4	7.11	6.67	105.0
	0.9	5.09	4.46	98.5
	1.3	4.24	2.67	99.7
8H-alprazolam (**III**)	0.3	7.01	5.92	108.3
	0.6	4.16	3.33	102.9
	0.9	3.56	2.59	101.4

tional concentrations in the linear range were established from intra- and inter-assay replicate analyses (Table 1). For within-day determination, each concentration studied was analyzed in replicates of five. For between day determination, samples were analyzed in triplicate in three batches processed on separate days. The mean of each batch was then used to determine the precision. The mean, standard deviation and relative standard deviation (RSD) were calculated at each concentration for **II** and **III**, respectively. (Table 1) The results showed that the inter- and intra-assay accuracy and precision were in agreement with accepted validation procedures.

The sensitivity of the method was evaluated by using samples of compounds **II** and **III** in the range $0.1–1.0 \times 10^{-4}$ M, each concentration was analyzed by triplicate**.** The detection (LOD) and quantitation (LOQ) limits, were 1.2 and 3.0×10^{-5} M, respectively for **II**, and 0.8 and 2.2×10^{-5} M, respectively for **III**. The specificity of the method was tested by spiking alprazolam solutions, in known samples of **II** and **III**: no interference was detected, as expected, since alprazolam shows no fluorescence under the assay conditions.

3.4. Applications to real cases

Commercial tablets containing 1.0 mg of alprazolam, were irradiated for 30 days out of the blister packaging. After irradiation, the tablets were dissolved in methanol by sonication for 30 min, filtered, and the filtrate was evaporated to dryness under reduced pressure. Determination of alprazolam content by the reported HPLC method [20] showed a global 9.0% of degradation; the spectrofluorimetric assay amounted to a 5.5% of fluorescent products (compounds **II** and **III**), the rest of degradation likely corresponds to compound **IV** which shows no fluorescence under the present conditions. On the other hand, commercial tablets containing 0.5 and 1.0 mg of alprazolam, that have been exposed to natural sunlight irradiation in their blister packaging during 792 and 605 days, respectively, were worked up as described. The assays showed fluorescence that corresponded to a range of 1.1–1.5% photodegradation.

4. Conclusions

Alprazolam was found to be photoreactive when exposed to UV radiation, consequently adequate light protection should be adopted for its storage and handling. Since the formation of the main photodegradation products is not detected by the currently used methods for alprazolam determination, a fluorimetric spectrophotometric assay was developed that allows quantitative determination of the two main photodegraded. Low pH's should be preferably avoided in preparations using alprazolam since the present study demonstrates that photolability increases in acidic medium. The photosensitivity observed in some patients treated with alprazolam could be related to photorearrangements leading to the formation of a six-membered condensed ring product, that exhibit an intense fluorescence. The proposed spectrofluorimetric assay allows determination of fluorescent products in concentrations as low as 10^{-5} M.

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References

[1] V. Andrisano, R. Gotti, A. Leoni, V. Cavrini, J. Pharm. Biomed. Anal. 21 (1999) 851–857.
[2] A. Albini, E. Fasani, in: A. Albini, E. Fasani (Eds.), Drugs: Photochemistry and Photostability, Royal Society of Chemistry, Cambridge, 1998, pp. 1–73.
[3] D.E. Moore, in: H.H. Tonnesen (Ed.), Photostability of Drugs and Drug Formulations, Taylor and Francis, London, 1996, pp. 9–38.
[4] H.H. Tonnesen, in: H.H. Tonnesen (Ed.), Photostability of Drugs and Drug Formulations, Taylor and Francis, London, 1996, pp. 1–7.
[5] J.T. Piechocki, in: A. Albini, E. Fasani (Eds.), Drugs: Photochemistry and Photostability, Royal Society of Chemistry, Cambridge, 1998, pp. 247–271.

[6] G.M.J. Beijersbergen van Henegouwen, Adv. Drug Res. 29 (1997) 79–170.
[7] C.E. Wright, T.L. Sissin, J.C. Fleishaker, E.J. Antal, J. Clin. Pharmacol. 37 (1997) 321.
[8] N.S. Nudelman, R.G. Waisbaum, J. Phys. Org. Chem. 10 (1997) 97–106.
[9] N.S. Nudelman, R.G. Waisbaum, J. Pharm. Sci. 84 (1995) 208–211.
[10] N.S. Nudelman, R.G. Waisbaum, J. Pharm. Sci. 84 (1995) 998–1004.
[11] N.S. Nudelman, C. Gallardo, Vitae 6 (1999) 29–40.
[12] N.S. Nudelman, R.G. Waisbaum, C. Gallardo, Atualidades de Fisico-Quimica Organica, (1998) 478–497.
[13] L.E. Allen, M.A. Erickson, Am. J. Health Syst. Pharm. 55 (1998) 1915–1920.
[14] M.R. Caira, B. Easter, S. Honiball, A. Horne, L.R. Nassimbeni, J. Pharm. Sci. 84 (1995) 1379–1384.
[15] R. Andersin, M. Mesilaakso, J. Pharm. Biomed. Anal. 13 (1995) 667–670.
[16] N.L. Stemm, J.W. Skog, R.H. Robins, Pharm. Res. 12 (1995) 738–745.
[17] M.J. Cho, T.A. Scahill, J.B. Hester, J. Pharm. Sci. 72 (1983) 356–362.
[18] M. Konishi, K. Hirai, Y. Mori, J. Pharm. Sci. 71 (1982) 1328–1334.
[19] R.M. Jiménez, E. Dominguez, D. Baía, R.M. Alonso, F. Vicente, J. Heteroc. Chem. 24 (1987) 421–424.
[20] USP DI, 16° edición, (1996) 38–39.
[21] D.J. Crouch, D.E. Rollins, D.E. Canfield, D.M. Andrenyak, J.E. Schulties, J. Anal. Toxicol. 23 (1999) 479–485.
[22] K.M. Hold, D.E. Rollins, D.J. Crouch, D.G. Wilkins, D.E. Canfield, R.A. Maes, J. Mass Spectrometry 31 (1996) 1033–1038.
[23] M.A. Hall, A. Robinson, R.M. Brissie, J. Anal. Toxicol. 19 (1995) 511–513.
[24] K.M. Hold, D.J. Crouch, D.E. Rollins, D.G. Wilkins, R.A. Maes, Forensic Sci. Int. 84 (1997) 201–209.
[25] D.D. Hong, M. Shah, in: J.T. Carstensen, C.T. Rhodes (ed.), Drug stability, principles and practices, 3 edn., Marcel Dekker Inc., New York, pp. 329–384.
[26] D.E. Moore, in: H.H. Tonnesen (Ed.), Photostability of Drugs and Drug Formulations, Taylor and Francis, London, 1996, pp. 63–82.
[27] Y. Harth, M. Rapoport, Drugs Sal. 14 (1996) 252–259.
[28] C.G. Reid, Can. Pharm. J. 128 (1995) 31–56.
[29] Y. Harth, M. Rapoport, Drugs Sal. 14 (1996) 252–259.
[30] C.G. Reid, Can. Pharm. J. 128 (1995) 31–56.
[31] L. Pazzagli, R. Banfi, G. Borselli, M.V. Semmola, Pharm. World Sci. 20 (1998) 136.
[32] Y. Watanabe, A Kawada, Y. Ohnishi, S. Tajima, A. Ishibashi, J. Am. Acad. Dermatol. 40 (1991) 832.
[33] A.J. Kanwar, R. Gupta, S. Das Metha, Dermatologica 181 (1990) 75.
[34] N.S. Nudelman, C. Gallardo, J. Pharm. Sci., 91 (2002) in press.
[35] O. Minoru, T. Tsutomu, N Toshihide, J. Pharm. Sci. 84 (1995) 786–790.