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Kinetic and mechanistic studies on the hydrolysis and photodegradation of diazepam and alprazolam†

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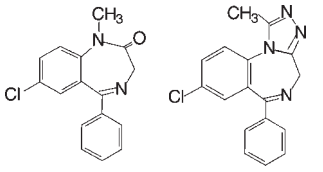
ABSTRACT: The corresponding *o*-aminobenzophenones are usually reported as the main degradation products in the hydrolysis of 1,4-benzodiazepinones. Nevertheless, in previous studies of diazepam (**DZ**) in acidic aqueous medium we isolated and characterized seven unexpected degradation products. Kinetic measurements under several reaction conditions shed some light on the mechanisms of the complex reactions that are taking place, and photochemical studies give hints on the mechanisms of chlorination and annelation processes. Alprazolam (**AL**) seems to exhibit an unusually high stability against hydrolysis under several conditions; nevertheless, the structure is sensitive to photolytic cleavage. The photodegradation of **AL** was studied in aqueous and methanolic solutions. Characterization of the isolated products by ^{1}H and ^{13}C NMR and mass spectrometry revealed that electron transfer, oxidation and rearrangement reactions take place. The influence of several variables such as pH, solvent composition and light irradiation were examined and mechanisms for the formation of three photoproducts are proposed. A specific method for the determination of **AL** in the presence of photoproducts was developed, which allowed kinetic determinations of the photostability of **AL**. The photosensitivity observed in some patients treated with **AL** seems to be due to one of the characterized photoproducts. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: diazepam; alprazolam; drug stability; hydrolysis; photostability; anxiolytics; benzodiazepinone pharmaceuticals; photosensitivity

INTRODUCTION

Diazepam (**DZ**) and alprazolam (**AL**) are among the most frequently prescribed benzodiazepinones (BDPs), which belong to an important class of drugs that exhibit different therapeutic applications such as anxiolytics, anticonvulsants, antithrombotics, HIV Tat antagonists, antitumor antibiotics, antipsychotics, anticonvulsants, hypnotics and muscle relaxants with different durations of action.[1] Recent studies carried out with **DZ** show that it also inhibits shock-induced ultrasonic vocalization in adult rats,[2a] while studies with **AL** demonstrate that a non-rewarding dose of **AL** potentiates the affective response to heroin in laboratory animals.[2b] Recently it was reported that **AL** was involved in the inhibition of monocyte chemoattractant protein.[3]



Since they are so widely used for the treatment of a wide range of clinical disorders, several sensitive methods are currently being developed for their quantitative determination in many matrices. Gas chromatography–ion trap tandem mass spectrometry was recently reported for the simultaneous detection of 22 BDPs.[4] In the particular case of **DZ** and **AL**, reversed-phase liquid chromatography–electrospray ionization mass spectrometry[5] and liquid chromatography–tandem mass spectrometry (LC–MS–MS)[6] have been described as especially suitable for the accurate determination of these drugs and their metabolites in biological matrices such as plasma and hair.

Photosensitivity associated with BDP has recently been reported,[7,8] particularly in patients treated with **AL**.[9] The ICH Harmonized Tripartite Guideline[10] and the recent FDA draft guidance[11] state that light testing should be an integral part of stress test. Therefore, it was of interest to test the stability of **DZ** and **AL** under both hydrolytic and photochemical stress conditions.

EXPERIMENTAL

Diazepam was obtained from Roche, and used as received. 2-(*N*-Methyl)amino-5-chlorobenzophenone (**1**),

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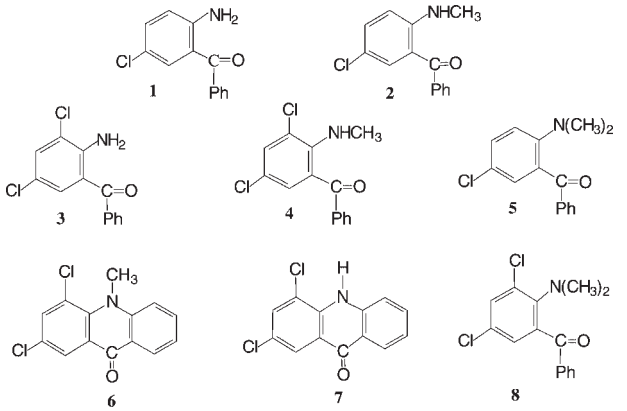


2-amino-5-chlorobenzophenone (**2**) 2-*N*-dimethylamino-5-chlorobenzophenone (**3**), 2-amino-3,5-dichlorobenzophenone (**4**), 2-(*N*-methyl)amino-3,5-dichlorobenzophenone (**5**), 2,4-dichloro-10-methylacridin-9-(10*H*)-one (**6**) and 2,4-dichloroacridin-9-(10-*H*)-one (**7**) were synthesized as previously described.[12] 2-*N*-Dimethylamino-3,5-dichlorobenzophenone (**8**) was identified and characterized in the reaction mixture by GC–MS. **AL** and the synthetic intermediates were obtained from Gador 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 and 5.0) were prepared according to standard methods. Solvents were purified according to described procedures.[13]

Mass spectra were recorded on a VG (Manchester, UK) Trio-2 mass spectrometer. High-resolution mass spectra were measured on a BG ZAB-SEQ4F mass spectrometer. NMR spectra were recorded on a Bruker (Karlsruhe, Germany) AM-500 spectrometer. NMR samples were dissolved in chloroform-*d* (Aldrich) and NMR spectra were referenced using tetramethylsilane as internal reference. HPLC experiments were carried out on a Hewlett-Packard (now Agilent Technologies) (Avondale, PA, USA) 1100 HPLC system consisting of an HP-G1311A Quat pump, HP 61315A UV detector and LiChrosorb RP-8 (5 μm) reversed-phase HPLC column (200×4.6 mm i.d.). Data acquisition and treatment of GC and HPLC experiments were carried out using a Hewlett-Packard HP-G2170AA Chem Station.

Quantitative GC determinations were carried out with Model 5890 Series II Plus system (Hewlett-Packard) with flame ionization detection (FID), using dried nitrogen as carrier gas and an HP5 (5% phenyl, 95% dimethylpolysiloxane) capillary column. A typical temperature programme that allowed the separation of the *o*-aminobenzophenones was a range from 170 to 180 °C at 2 °C min^{-1}, then from 180 to 200 °C (held for 10 min) at 0.5 °C min^{-1}. Retention times, t_r (min) ± 0.03, were as follows: **5**, 12.00; **1**, 13.00; **2**, 15.48; **3**, 16.05; **4**, 18.00; and **8**, 16.80. The products in the reaction mixtures were identified by CG–MS and NMR of the compounds isolated by column chromatography; then the GC retention times were checked against authentic samples independently synthesized, as described previously.[12] Acridinones **6** and **7** were determined by analytical TLC, using silica gel G60 plates with 90% toluene–cyclohexane as eluent ($R_F = 0.11$ and 0.28 for **6** and **7,** respectively) and compared with authentic samples independently prepared. Semi-quantitative determinations were carried out by UV-visible spectrophotometry.

For kinetic measurements, the corresponding substituted aminobenzophenones (3×10^{-3} M) were dissolved in methanol and appropriate volumes of dilute HCl were added to obtain concentrations of 0.5, 1.0 and 1.5 M in HCl in methanol–water (84:16 and 1:1). The ampoules were immediately placed in a thermostat at 80 ± 0.1 °C. Ampoules were taken at appropriate time intervals and aliquots were worked up with CH_2Cl_2 by standard procedures and analysed by GC.

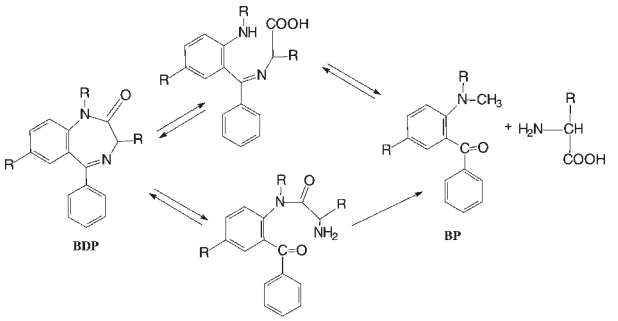
The reactions were run under pseudo-first-order conditions using a 100-fold excess (at least) of HCl over the substrate. The reactions were followed for at least three half-lives and the final values were determined after more than 10 half-lives.

For light-stress exposure testing, a photoreactor provided with an HPA-400 W medium–pressure metal halide lamp (Philips) and mirrors was used. This metal halide lamp has a close resemblance to sunlight and the output spectrum is fairly uniform across the 350–650 nm region.[14] The photoreactor geometry and the methods for the calibration of the lamp intensity were reported previously.[15]

RESULTS AND DISCUSSION

Mechanism of hydrolysis

The diazepinone seven-membered ring in BDP is known to be highly sensitivity to hydrolysis in both acidic and basic media. The ring opening can occur by initial breakage of the amide functionality or the C═N bond, in both cases the final product being the substituted 2-aminobenzophenone (BP, Scheme 1). Most of the



Scheme 1

reported methods for stability studies of BDP are based on the determination of these very well characterized compounds.

Previous studies from our group on the reaction of **DZ** with HCl in aqueous MeOH media showed that, in addition to the expected 2-methylamino-5-chlorobenzophenone (**1**), other products, **2–8**, were formed. Formation of products **1–3** in MeOH–HCl media is easily explained, but the products **4–8** were completely unexpected and a careful isolation and full characterization was therefore undertaken and reported.[12]

Products **4–8** were thought to arise from further reactions of products **1** and **2**, and in order to provide some clues about the routes of formation of these rare products, kinetic studies of the reactions of **1** and **2** with HCl at different concentrations in MeOH–H_2O (1:1) were carried out and reported.[16] The disappearance of the starting 2-aminobenzophenone, **1** or **2**, and the appearance of the reaction products was followed by GC of the reaction mixtures and the rate constants for the reactions giving rise to the different products were calculated. Similar studies were then carried out at higher MeOH content. Figure 1 shows a representative plot for the reaction of 2-(*N*-methyl)amino-5-chlorobenzophenone with 1.0 M HCl in methanol–water (84:16) at 80 °C.

In contrast, **AL** was insensitive to acid or base hydrolysis. The aromatic triazole ring seems to inhibit the hydrolytic ring opening. Even after reflux at very low pH

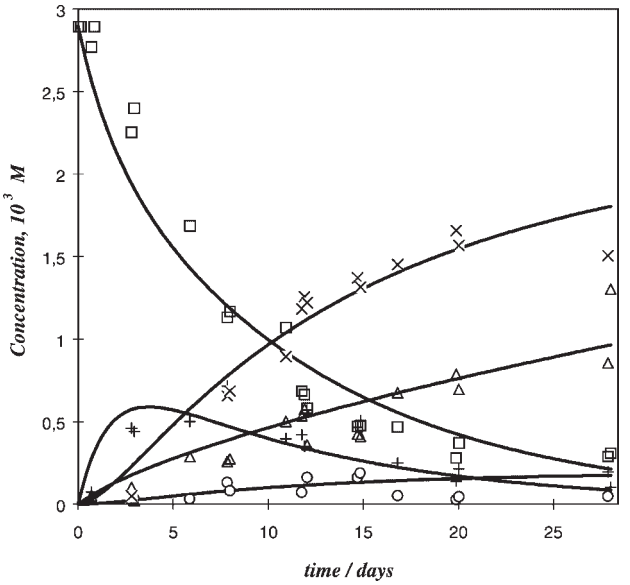


Figure 1. Reaction of 2-methylaminobenzophenone (**1**) with 1.0 M HCl in methanol:water (84:16) at 80 °C. Data as a function of time for (□) the disappearance of **1** and the formation of (+) **2**; (×) **4**; (○) **5**; and (△) **3**

(~2) or at high pH (9–11), **AL** was recovered unchanged. Only by NMR analysis of the reaction mixture signals was it possible to detect the presence of the substituted aminobenzophenone (see Fig. 2).

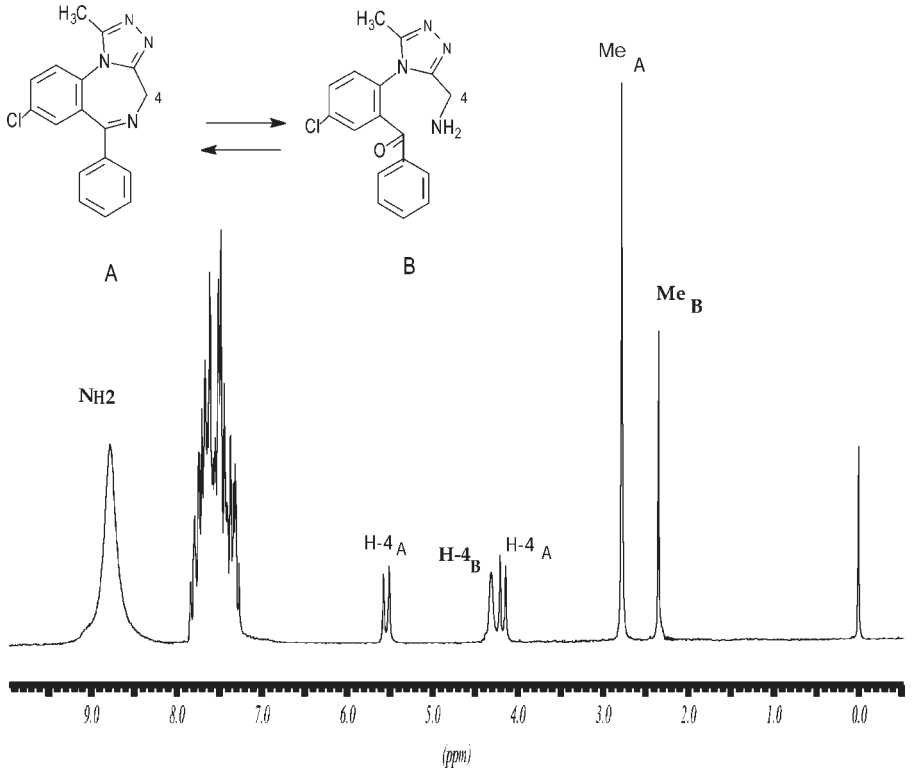


Figure 2. ^{1}H NMR spectrum of the reaction mixture of **A** at pH ≈ 2. The signal for the protonated amino group of the corresponding BP (B), the singlet for the methylene group H-4_B (between the double doublet for H-4_A) and the singlet for the protons of the Me_B group are clearly seen

These results suggest that the ring opening is a reversible reaction and the starting structure of **AL** was recovered intact in the attempted isolation of the hydrolysis products. Similar results were observed when accelerated thermal degradation was attempted. The compound was then exposed to photochemical stress.

Photochemical studies

The stress studies carried out on **AL** by UV irradiation revealed that the photolability of the drug is the most adverse stability factor. The main photodegradation products were isolated and characterized as 1-methyl-6-phenyl-4*H*-*s*-triazo-[4,3-α][1,4]benzodiazepinone (8-hydroalprazolam) (**9**), 5-chloro[5″-methyl-4*H*-1,2,4-triazol-4-yl]benzophenone (**10**) and triazolaminoquinoleine. Accelerated pH-dependent studies show that the photolability increases as the pH decreases; at pH 9.0 photodegradation does not occur; therefore, photochemical degradation studies of **AL** were carried out in acidic media. The rate of reaction was followed by a spectrofluorometric method specially developed for studying the stability of **AL** tablets under photochemical stress.[17] Table 1 lists the rate coefficients of the photodegradation of **AL** in buffered solutions at pH 2.0, 3.6 and 5.0.

The reaction rate constants were calculated using a first-order rate equation. The reactions were followed at least for four half-lives and, as shown in Table 1, the largest rate coefficient was observed at pH 2.0.

The rate of disappearance of **AL** and the rate of appearance of the main photodegradation products were followed by HPLC. Figure 3 shows a typical plot for the reaction in pH 3.6 buffer solution. The photodegradation of **AL** gives triazoloquinoleine as the main degradation product. This compound exhibits high fluorescence and might be the responsible for the photosensitivity observed in some patients treated with **AL**. 8-Hydroalprazolam (**9**) is the second in importance, followed by the triazolobenzophenone. Usually, the substituted aminobenzophenones are the main hydrolytic degradation products of 1,4-benzodiazepinones.

Some photodegradation studies were also carried out on **DZ**. In relation to the unexpected hydrolytic behaviour described above, reactions of 2-methylaminobenzophenone were carried out under photochemical stress in HCl

Table 1. Photodegradation of **AL** in aqueous media: pseudo-first order rate coefficients[a,b]

pH	k_{obs} (10^{-5} s^{-1})	t (h)
2.0	3.61	5.3
3.6	1.67	11.5
5.0	0.55	34.7

[a] [Alprazolam] = 2.75×10^{-3} M in phosphate–citrate buffer.
[b] Spectrofluorometric method (λ_{exc} 260 nm, λ_{em} 435 nm).

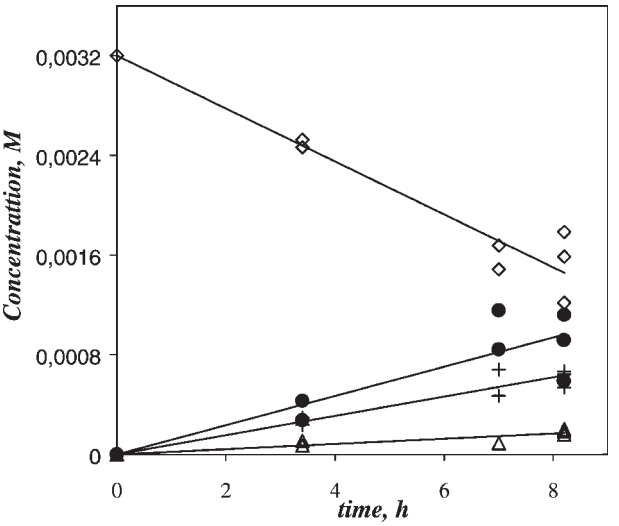


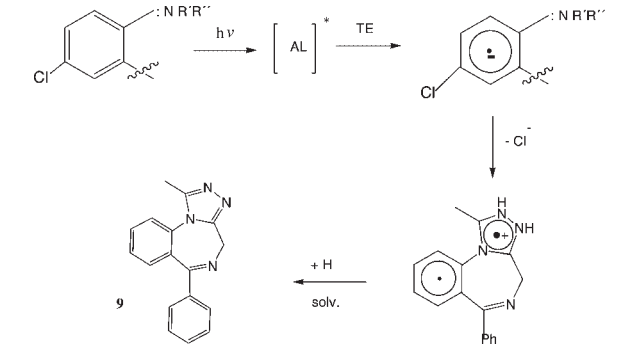
Figure 3. Photodegradation of **AL** in pH 3.6 buffer. Concentrations measured by HPLC as a function of time: (◇) alprazolam; (+) 8-hydroalprazolam; (●) triazolquinoleine; (△) triazolbenzophenone

in aqueous methanol media, using similar techniques to those described above for **AL**. In these cases, the reactions were carried out in sealed ampoules. The experimental results allowed us to conclude that degradation is accelerated by photochemical stress. Gas evolution was observed which was identified as molecular hydrogen, by the following test using Dräger tubes (Drägenwerk, Germany): (a) with a catalyst of the reaction $H_2 + O_2 \rightarrow H_2O$ and a moisture indicator, a blue colour was observed (hydrogen detection); (b) no colour development was observed with *o*-tolidine, which led to a yellow–orange product with chlorine.

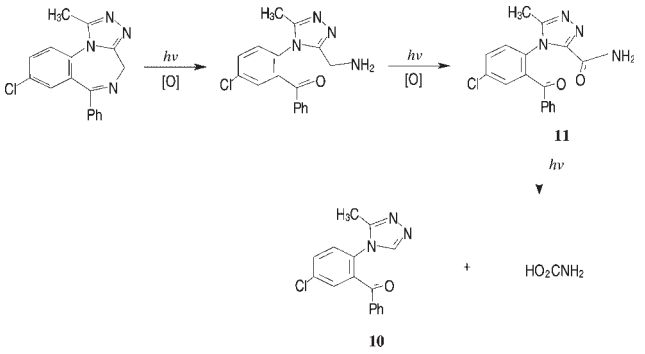
Mechanism of formation of degradation products

For **AL**, the simplest photodegradation observed is dechlorination to give the 8-hydroalprazolam. Dehalogenation of aromatic compounds is a well-known reaction in the photochemistry of aryl halides, and occurs via by an electron transfer mechanism between a donor and an acceptor (the aryl halide).[18]

The mechanism of the reaction can be intermolecular or intramolecular if the donor and the acceptor are in the same molecule.[19,20] Only a few studies have been carried out with drugs with clinical activity, but diclofenac undergoes dechlorination[21] and midazolam defluorination[22] under photolytic stress. Nitrogen atoms are known to be very good electron donors and since **AL** has four nitrogen atoms, an intramolecular electron transfer can be postulated to occur with any of them. Scheme 2 shows a plausible mechanism for electron transfer from the nitrogen bond to the chlorinated ring showed as: NR′R″ in Scheme 2, where [**AL**]* is used to indicate **AL** in the



Scheme 2



Scheme 3

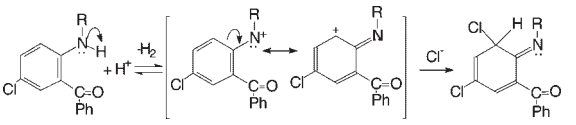
excited state. Detachment of a chloride ion followed by proton abstraction from the solvent gives product **9**.

For the formation of the triazolbenzophenone under photochemical stress, photooxidative opening of the diazepinone ring is postulated followed by detachment of a carbamic HO_2CNH_2 molecule. Although this type of reaction has not been observed with simpler BDPs, it is likely that the additional stabilization given by the aromatic triazole ring could be a driving force for the operation of this route. The isolation as traces of product **11** supports the proposed mechanism (Scheme 3).

A plausible mechanism for the formation of the main degradation product, the fluorescent triazolaminoquinoline, can be postulated: a similar opening of the diazepinone ring (as in Scheme 3) followed by reaction between the activated methylene and the carbonyl group, giving rise to a highly stabilized six-membered ring.

Finally, all the observations on the photochemical reactions of 2-methylaminobenzophenone in HCl in aqueous MeOH media offer an explanation for the formation of the unexpected products observed in the acid hydrolysis of **DZ**. Although somewhat speculative, a nitrenium intermediate could account for the formation of the double chlorinated products and the acridinones as shown in Scheme 4.

The elimination of molecular hydrogen from the corresponding aminobenzophenone would give an unstable nitrenium intermediate that could react with chloride ions in excess giving the 3,5-dichloro intermediate. This



Scheme 4

unstable intermediate could be stabilized to a 3,5-dichloroaminobenzophenone (**4** or **5**) or react with the *o*-hydrogen of the second phenyl ring affording 3,5-dichloroacridinones (**6** or **7**). Consistent with this mechanism is the fact that no monochlorinated acridinones were observed and also that the formation of nitrenium ion from amines is known to be increased by UV irradiation.[18] (Since the nitrenium ions are extremely unstable and difficult to generate,[23] one of the referees proposed an alternative concerted TS for both the molecular hydrogen elimination and chloride ion attack. This mechanism may equally account for the obtained results; we prefer the mechanism proposed in Scheme 4 on the basis of the effects observed by UV irradiation.)

CONCLUSIONS

The isolation of the degradation products and the kinetic determinations under photochemical stress shed light on the photostability of **AL** and **DZ** and the mechanisms of formation of the degradation products. The results indicate that light exposure and acidic media should be avoided during the process formulation and handling of **AL**. When studying acid hydrolysis of diazepam, not only formation of the expected 2-(*N*-methylamino)benzophenone should be monitored but also the other products described in this paper.

Acknowledgements

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