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# Isolation, identification and determination of the major degradation product in alprazolam tablets during their stability assay

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**Abstract**

The presence of a degradation product of alprazolam tablets that emerged throughout a short-stability assay has been determined and properly characterized. For this purpose an efficient methodology has been successfully applied, including SPE and HPLC methods for isolation and purification, respectively. LC/MS, MS/MS, $^{1}$H NMR, $^{13}$C NMR, UV and IR have been employed for structural elucidation confirming the identity of this impurity as 7-chloro-1-methyl-5-phenyl-[1,2,4]triazolo[4,3-a]quinolin-4-amine or triazolaminoquinoleine.

The impurity, previously described as a long-term photodegradation product of alprazolam active pharmaceutical ingredient, was rapidly formed in the absence of light, but required the presence of excipients and its rate of formation increased with heat and humidity.

In addition, a LC method has been developed and validated including triazolaminoquinoleine for the adequate determination of alprazolam and its mayor degradation product in tablets as pharmaceutical forms.


*Keywords:* Alprazolam; Impurity identification; Stress test; Triazolaminoquinoleine

## 1. Introduction

Stability testing is the primary tool used to assess expiration dating and storage conditions for pharmaceutical products. Many protocols have been used for stability testing, but most in the industry are now standardizing on the recommendations of the International Conference on Harmonization (ICH). Stability testing includes long-term studies, where the product is stored at room temperature and humidity conditions, as well as accelerated studies where the product is stored under high heat and controlled humidity conditions. Stability studies are linked to the establishment and assurance of safety, quality and efficacy of the drug product. In order to assess stability, the appropriate physical, chemical, biological and microbiological testing must be performed. One of the evaluation criteria is the appearance of impurities [1].

Alprazolam is a benzodiazepine derivative that is currently used in the treatment of generalized anxiety, panic attacks with or without agoraphobia, and depression [2].

The corresponding stability testing of alprazolam tablets containing 0.25 mg of the active substance revealed about 15 μg of an unknown impurity per tablet. This result was obtained by HPLC with ultraviolet diode array detection following a previous HPLC method with minor modifications [3]. The impurity exceeded the 1% identification threshold (based on the <1 mg administration per day), which called for structural identification. HPLC retention time of the impurity did not coincide with any officially available standard of impurities. Nudelman et al. [4] performed previous studies on alprazolam thermal, hydrolytic and photochemical degradation products on the active pharmaceutical ingredient (API). The authors only obtained photostability related products after a long-term light exposure and only that route was studied with the tablets. However, the conditions of sample storage during the stability assay of alprazolam tablets in the present study (inside the blisters, packing material and closed climatic chambers) excluded photodegradation.

The purpose of this study was to identify this unknown impurity in alprazolam tablets and confirm the structure by chemical independent organic synthesis of this compound. NMR, IR, MS and LC–UV (DAD) techniques were employed for characterization. After that, a HPLC method was developed and validated

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for a rapid quantification of both alprazolam and the impurity in the tablets.

## 2. Experimental

### 2.1. Chemicals

Standard of alprazolam as well as tablets and excipients were kindly provided by CINFA S.A. (Pamplona, Spain). Acetic acid and ammonia (28%) were from Panreac (Barcelona, Spain), dimethylsulfoxide (DMSO) was from Scharlab (Barcelona, Spain), $H_3PO_4$ (85%), ammonium acetate and acetonitrile (HPLC grade) from Merck (Darmstadt, Germany), and water was purified with a Milli-Q plus system from Millipore (Bedford, MA, USA).

### 2.2. Standard solutions and sample preparation

In all cases the diluent for standards and samples was the mobile phase. Alprazolam and triazolaminoquinoleine standards solutions were prepared by exactly weighing around 5.0 mg of each separately in 100 mL volumetric flasks.

Two formulations were analyzed: one containing 0.25 mg alprazolam and the another one with 2 mg of alprazolam per tablet. For quantification both samples were diluted to a final theoretical concentration of 0.05 mg alprazolam/mL.

### 2.3. Stability conditions

The stability study was performed with tablets kept in their blisters (PVC-PVDC/Aluminium) and stored in climatic chambers under the following conditions of temperature and relative humidity (R.H.): chamber A: 25 °C and 60% R.H., chamber B: 30 °C and 60% R.H. and chamber C: 40 °C and 75% R.H. Samples were analyzed at 0, 3 and 6 months.

### 2.4. HPLC analysis

The HPLC method was similar to one previously described [3], with slight modifications. LaChrom Elite HPLC system from VWR consisted of a quaternary pump, an automatic injector, a variable wavelength detector and a column oven. The column was ODS Hypersil (Hewlett Packard, Las Rozas, Madrid, Spain) 200 mm × 4.6 mm and 5 μm. It provided baseline separation with isocratic conditions at pH 4.2 in less than 12 min for the impurity and alprazolam. The mobile phase consisted of buffer A/Acetonitrile 45:55 (v/v), buffer A being: 25 mM $KH_2PO_4$ brought to pH 4.2 with $H_3PO_4$. The flow-rate was 0.75 mL/min and the injection volume was 20 μL. The oven temperature was set at 40 °C and UV detection was performed at 234 nm.

### 2.5. LC/MS analysis

Experiments were carried out on an Agilent 1100 LC/Esquire 3000 (Bruker). MS spectrometer was equipped with Electrospray ion source and ion trap mass analyzer. The chromatographic conditions were: a ODS Hypersil 200 mm × 4.6 mm, 5 μm column (Hewlett Hackard, Las Rozas, Madrid, Spain), kept at 40° C. The mobile phase was 25 mM ammonium acetate at pH 4.2/acetonitrile, 45/55 (v/v). UV detection was performed at 234 nm, and the injection volume was 20 μL. The MS optimized parameters were: positive polarity; drying gas flow and temperature 6 L $min^{-1}$ and 300 °C, respectively, and nebulizador pressure, 16 psi.

### 2.6. SPE isolation

For the isolation by SPE, Waters® Oasis HLB (6 g) LP Extraction Cartridges were used. The mobile phase was 10 mM ammonium bicarbonate at pH 4.2 (A) and acetonitrile (B). 59.42 g of sample were dissolved in 195 mL of DMSO. The mixture was centrifuged at 4000 rpm during 10 min in order to eliminate the insoluble excipients and the supernatant was concentrated in a rotary evaporator (Rotavapor® R-114, Flawil, Switzerland). The concentrate was made up to 27 mL final volume and distributed in 9 cartridges, in order to load 3 mL in each one. Cartridges were eluted in parallel in eighteen steps. The collected fractions were concentrated in a rotary evaporator to dryness.

### 2.7. HPLC isolation

For the fine isolation of the degradation product an Agilent Technologies 1100 Series HPLC provided with an automatic injector, a diode array detection system and a column oven was used. The chromatographic method consisted of: a ZORBAX® ODS Semi-preparative column (Hewlett Packard, Las Rozas, Madrid, Spain) 250 mm × 9.4 mm; 5 μm, kept at 40° C. The mobile phase was 25 mM ammonium acetate at pH 4.2/acetonitrile, 45/55 (v/v). The flow rate was 3.5 mL/min and the injection volume was 100 μL. UV detection was performed at 234.

### 2.8. MS and MS/MS analysis

MS/MS experiments were carried out on the isolated and the synthesized compound with the aforementioned mass spectrometer in the same conditions performed for the LC/MS analysis by direct injection with a Cole Palmer 74900 series syringe pump (Vernon Hills, IL, USA) at a flow-rate 240 μL/h.

### 2.9. IR analysis

IR experiments were performed with a FT-IR spectrometer (Perkin-Elmer Spectrum 2000, Wellesley, MA, USA) on the isolated and the synthesized compound. Data acquisition from 4000 $cm^{-1}$ to 370 $cm^{-1}$. A 1% (w/w) pressed discs were prepared, employing KBr as transparent material.

### 2.10. NMR analysis

NMR experiments were acquired on a NMR spectrometer (SPECTROSPIN and BRUKER 300 MHz/52 mm, Milton, Ontario, Canada) in $CDCl_3$ at 298 K. $^1H$ and $^{13}C$ spectra were

acquired using 64k and 32k data points, respectively. The proton chemical shifts were referenced to the TMS signal at 0 ppm (25 °C) and the carbon chemical shifts to the $CDCl_3$ signal at 77 ppm (25 °C).

### 2.11. New HPLC method validation

After the optimization of several parameters, the method was validated with a Discovery $C_8$ column, 5 μm particle size, 150 mm × 4.6 mm from Supelco (Madrid, Spain). It provided baseline separation with isocratic conditions at pH 7.0 and a run time of under 3 min. The mobile phase consisted of Buffer A/acetonitrile 43:57 (v/v), buffer A being: 25 mM $KH_2PO_4$ brought up to pH 7.0 with KOH. Flow rate was 1.5 mL/min and UV detection was performed at 234 nm. The injection volume was 20 μL and oven temperature was kept at 40 °C. Standards and samples were prepared to a final theoretical concentration of 0.04 mg alprazolam/mL.

The selectivity was tested by running solutions containing the excipients of the speciality in the same quantities and conditions as the samples to show that there was no peak at the retention time corresponding to the analytes.

Validation parameters were tested in two different ranges for alprazolam and triazolaminoquinoleine. The linearity was tested for alprazolam by preparing triplicate standard solutions at five concentration levels, from 75 to 120% of alprazolam concentration (30–50 μg/mL) and from 0.05 to 5% (0.02–2 μg/mL) for the impurity. For pharmaceutical studies in drug products one method for determining accuracy is the application of the analytical procedure to synthetic mixtures of the drug components to which known quantities of the drug substances to be analyzed have been added. It was tested in triplicate at three levels (80, 100 and 110%) and in parallel with the linearity assay. The percent recovery and the R.S.D. were then calculated.

Intra-assay precision data were obtained by repeatedly analysing, in one laboratory on one day, 10 aliquots of a homogeneous sample. All of them were independently prepared according to the method procedure and with the corresponding standards. Data for intermediate precision were obtained by repeating the intra-assay experiment on a different day with newly prepared solutions.

The limit of quantification (LOQ) for triazolaminoquinoleine was established by applying the EURACHEM method, where LOQ is calculated when R.S.D. value is equal to 10% [5]. However, this is just one approach, because the actual LOQ was established by validating the method to the lower level (0.05%). The detection limit (LOD) was calculated by means of the relation LOD = (3/10)LOQ and checked experimentally.

## 3. Results and discussion

### 3.1. HPLC

During the stability assay of alprazolam tablets, an unidentified degradation product appeared after 6 months in climatic chambers as described above. The compound showed a characteristic UV spectrum with maximum at 234 nm as can be observed in Fig. 1. Moreover, it showed increasing concentrations at increasing storage time, temperature and humidity. Simultaneously, a decrease in the peak of alprazolam was measurable in the tablets stored in climatic chambers at 40 °C and 75% R.H. The percentage in area reached 6% of the active compound after 6 months in some formulations and that made characterization necessary.

The low content (in total weight) of alprazolam in the tablet and, consequently, the small amount of degradation product present in the sample presented a challenge. For this reason an additional accelerated degradation study was carried out in order to generate a greater content in the target compound. Alprazolam API and a synthetic mixture tablet corresponding to 0.25 mg dose were subjected to two different treatments. In the first one, samples were put in to an oven at 105 °C for 6 h in opened vials. In the second one samples were exposed to humidity coming from water placed in the bottom of a container in the same conditions.

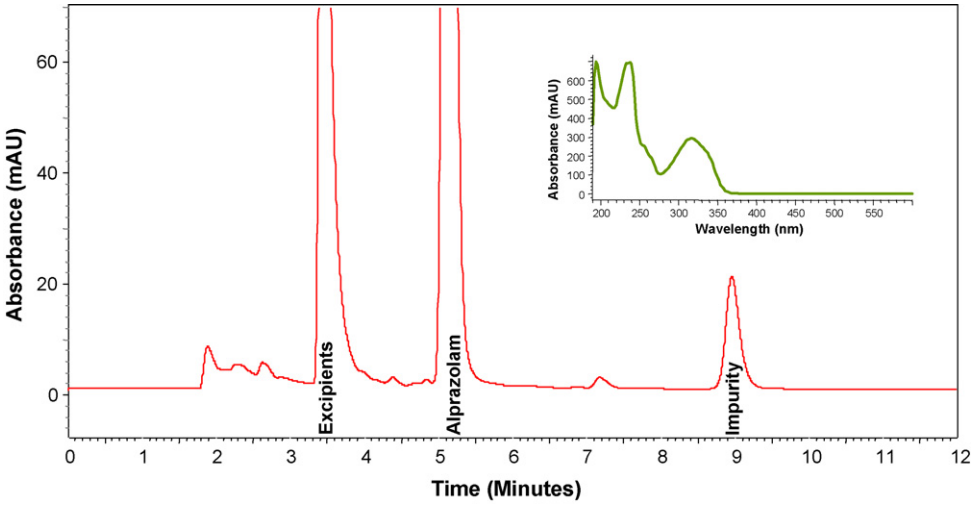


Fig. 1. Chromatogram showing alprazolam tablet coming from the stability assay after 6 months storage at 40 °C/75%HR and UV spectrum of the impurity. ODS Hypersil Column. Mobile phase: 25 mM phosphate buffer pH 4.2/ACN (45:55), at 0.75 mL/min, 40 °C. UV detection at 234 nm.

Table 1
Alprazolam content in the API and 0.25 mg tablets after stress tests referred to control (alprazolam content before treatment)

| | API | 0.25 mg tablet |
|---|---|---|
| Treatment 1 | 99.3 ± 0.2 | 100.4 ± 0.4 |
| Treatment 2 | 99.0 ± 0.4 | 86.7 ± 1.6 |

Treatment 1: 105 °C during 6 h in opened vials. Treatment 2: 105 °C during 6 h in closed container having water in the bottom. Results are expressed as mean ± standard error ($n = 3$).

The heat stress test results are summarized in Table 1. No statistical differences were observed for the API in the active substance content, either with or without humidity. However, in the formulation product prepared in the laboratory, alprazolam was degraded in the presence of humidity. Moreover, a new peak appeared showing the same retention time of the unknown degradation product, with a content of 1.00 ± 0.03% in area with respect to the original alprazolam content. As can be observed in the Fig. 2, the excipients, under the same degradation conditions, did not interfere with the new peak determination.

In accordance with the results observed in samples from the climatic chambers, temperature and humidity proved to play an important role in the generation of this degradation product. Moreover, in a new assay it was shown that even the humidity coming from the excipients was enough to promote the degradation when the stress test was developed in closed vials kept in a heated oven without humidity.

### 3.2. LC/MS

The first obvious tool to obtain chemical information was LC/MS, but the LC/UV method employed a non-volatile buffer

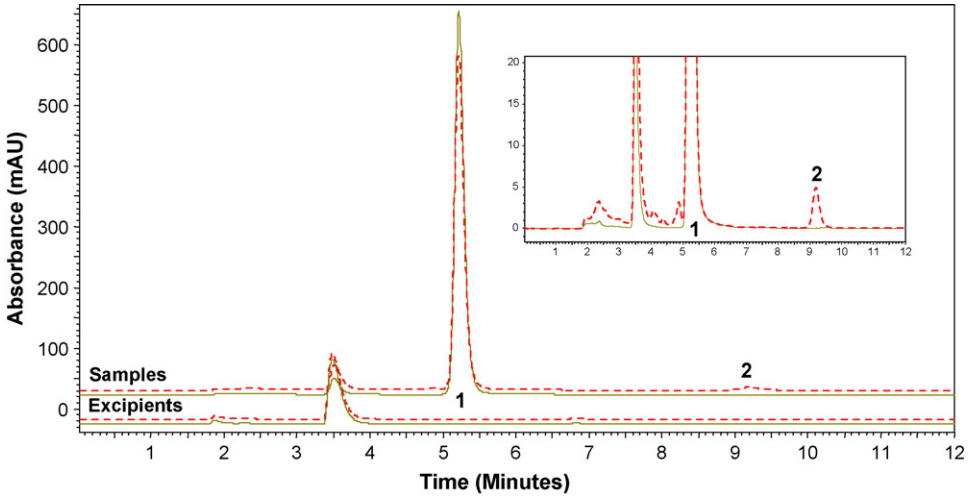


Fig. 2. Chromatograms corresponding to the accelerated degradation study: Excipients and the tablet corresponding to 0.25 mg dose before (continuous lines) and after treatment: 105 °C for 6 h in a closed container with water in the bottom (discontinuous lines). Chromatographic conditions: see Fig. 1. Peak identification: (1) alprazolam and (2) degradation product.

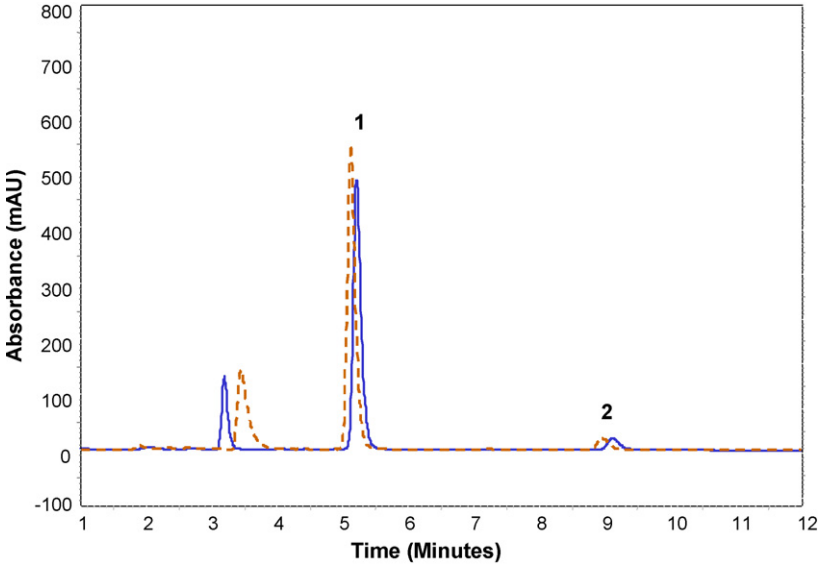


Fig. 3. Chromatograms corresponding to alprazolam tablet: (a) Mobile phase: 25 mM phosphate buffer pH 4.2/ACN (45:55) (discontinuous line); (b) Mobile phase: 25 mM ammonium acetate buffer pH 4.2/ACN (45:55) (continuous line). ODS Hypersil Column. Flow rate: 0.75 mL/min, 40 °C. UV detection at 234 nm. Peak identification: (1) alprazolam and (2) degradation product.

that could be substituted without changing the selectivity. Therefore, the 25 mM phosphate solution at pH 4.2 was substituted in the mobile phase for a 25 mM ammonium acetate buffer at pH 4.2 keeping the content of acetonitrile (55%). The result obtained (Fig. 3), revealed the equivalent selectivity offered by both mobile phases.

The next step was the optimization of the MS conditions that allowed the ionization and detection of the compound, and have been described in Section 2.4. As no fragmentation profile was achieved in MS, the structural information was difficult to assess. The molecular weight of the degradation product was 308 uma, showing a *m/z* of 309 $[M+H]^+$. Furthermore, this molecular ion presented a $[M+H+2]^+$ isotopic peak with 33% intensity showing the presence of one chloro in the structure.

In a previous study, Nudelman et al. [4] performed stress studies of alprazolam API under accelerated thermal, hydrolytic, and photochemical conditions, finding light to be the most adverse stability factor. The main photodegradation products, obtained after 408 days treatment, were isolated and characterized. One of them, 7-chloro-1-methyl-5-phenyl-[1,2,4]triazolo[4,3-a]quinolin-4-amine (triazolaminoquinoleine), showed the same molecular weight as the one obtained for the degradation product in the tablets either in climatic chambers or in the heat and humidity stress test.

### 3.3. SPE isolation

The degradation product quantity necessary for structural identification techniques was estimated at around 10 mg. To reach this objective tablets with a higher dose (2 mg per tablet) were used. 200 tablets were subjected to the degradation conditions previously described for 72 h.

The degradation product generated was around 20% of the initial alprazolam content, nevertheless this compound was still a minor component in the sample (∼0.15% in total weight). Therefore the isolation of the degradation product was achieved employing solid phase extraction (SPE) in order to obtain a rich fraction in the target degradation product.

The isolation methodology employed followed a similar scheme to that previously applied in our laboratory for a different analysis [6] and by others [7] and showed its usefulness as a general strategy.

Oasis HLB cartridges for solid phase extraction (SPE) were used because they permit the development of simple

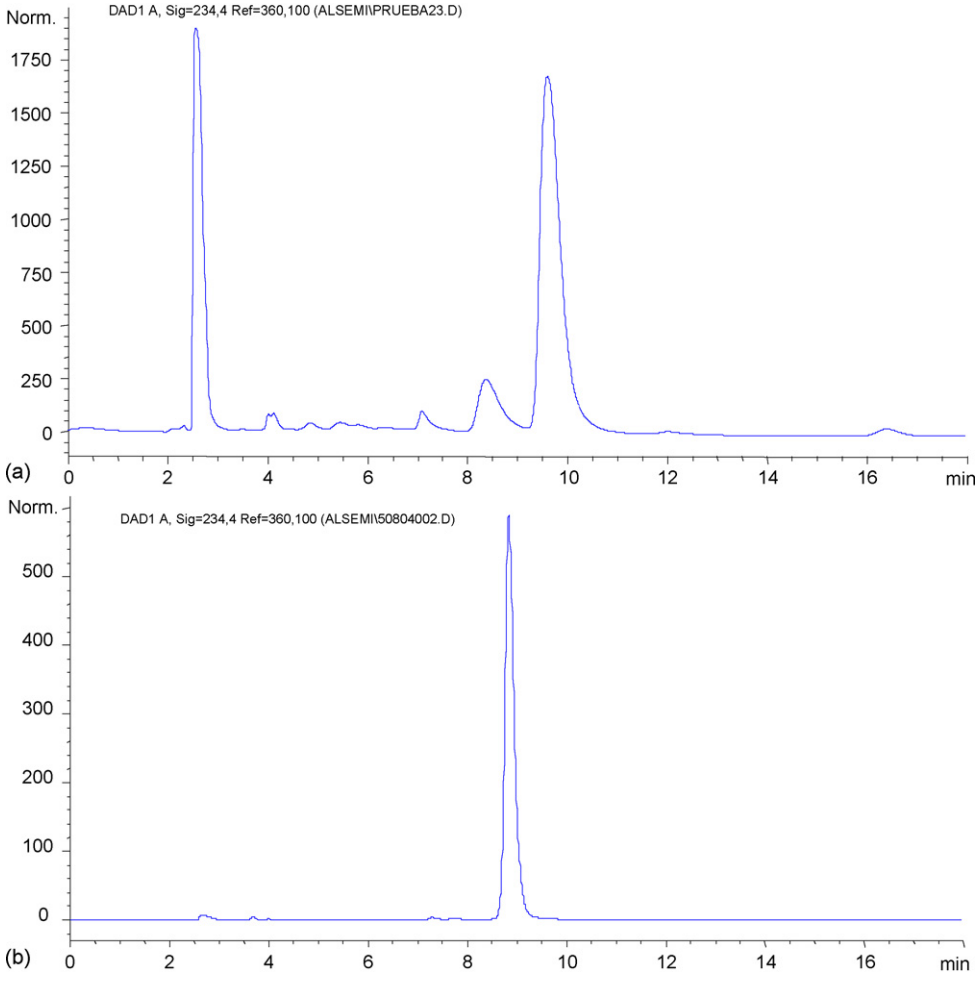


Fig. 4. (a) Chromatogram showing the semi-preparative HPLC separation of the solution obtained from the dried extract coming from the SPE; (b) chromatogram of the isolated degradation product analyzed with the analytical HPLC method.

methodologies. First, the elution order of the compounds was rather similar to those found in the HPLC method. Second, the extraction solvents were absolutely compatible with the chromatographic conditions allowing the direct injection of the collected fractions for their analysis by HPLC.

The mobile phase consisted of solution A (10 mM ammonium bicarbonate) and acetonitrile. After loading the sample, the cartridge was washed with 10 mL of Buffer A. The elution process was divided into two steps. Firstly, 15 volumes of 20 mL of eluotropic phase (Solution A/AcN 70:30 (v/v)) were used to eliminate entirely alprazolam content (Step I). Secondly, a total volume of 60 mL of acetonitrile distributed in 10, 25 and 25 mL corresponding to the 16th, 17th and 18th fractions (Step II) to extract the target compound. The fractions were analyzed by HPLC. More than 95% of the total impurity was found in 17th fraction and consequently the remaining fractions in Step II were discarded to avoid unnecessary contaminations. Subsequently, the acetonitrile was removed in the rotary evaporator obtaining a dried extract.

### 3.4. HPLC isolation

In order to obtain a pure compound a further purification step to SPE was required and semi-preparative HPLC was the technique selected for that purpose. The optimized analytical method was scaled to semi-preparative HPLC employing a ZORBAX® ODS Semipreparative 250 mm × 9.4 mm; 5 μm from Hewlett Packard kept at 40° C under isocratic conditions with 45% (v/v) 25 mM ammonium acetate at pH 4.2 and 55% (v/v) of acetonitrile at 3.5 mL/min. The separation profile is shown in Fig. 4.

The dried extract was re-dissolved in the minimum volume (2 mL) of DMSO and filtered through 0.45 μm nylon membrane. The injection volume applied was 100 μL and the recollection time was established from 9 to 10.5 min.

Finally, it was possible to isolate 9 mg of the degradation product free from interfering components. The chromatogram of the isolated compound showed a purity higher than 95%. Purity was also confirmed with the UV-HPLC method.

### 3.5. MS and MS/MS

To confirm the identity of the isolated product, MS and MS/MS experiments using an ion trap system were performed, in order to achieve a characteristic fragmentation profile. The purified compound was introduced by direct infusion and analyzed by ESI in the positive mode with *m/z* scan from 50 to 450. The spectrums are shown in Fig. 5.

The compound gave a molecular ion at *m/z* 309 [M+H] in accordance to the result obtained in LC/MS experiment prior to isolation.

When the parent ion *m/z* 309 was selected and fragmentized in the MS/MS experiment, it produced a fragment ion at *m/z* 268 that could be a possible elimination of acetonitrile (M-41) from the degradation product. This eliminated fragment is typical of quinolines and isoquinolines compounds. Furthermore, another fragment at *m/z* 233 appeared that could probably have been

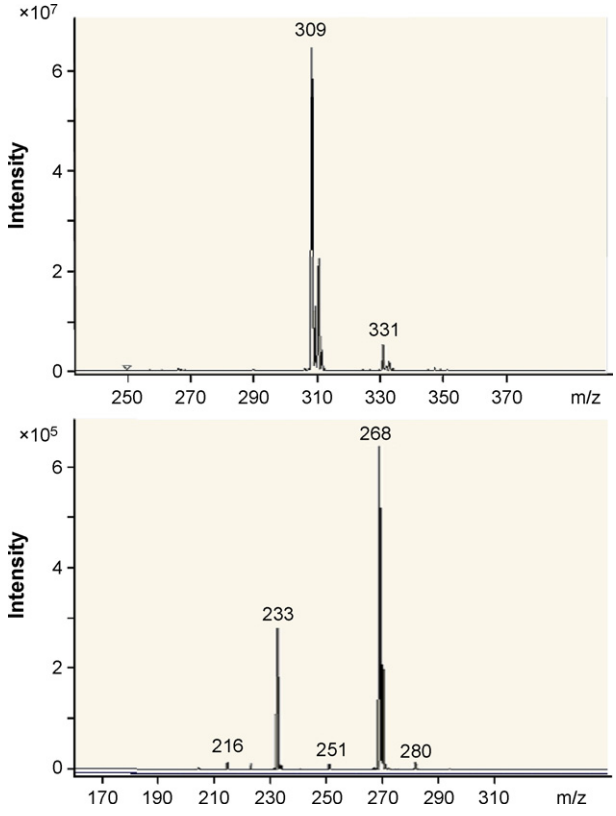


Fig. 5. MS (top) and MS/MS (bottom) spectrums. For chromatographic and mass spectrometry conditions see Section 2.

assigned as the loss of chloro from the first daughter ion, because of the difference of 35 mass units. The absence of a band at *m/z* 235 in a 1/3 relation supports this assignment.

### 3.6. IR

The Infrared spectrum of the isolated compound (Fig. 6) revealed two bands at 3460 and 3280 $cm^{-1}$ as a result of distinct vibrations modes of a N–H bond, assigned to $NH_2$ antisymmetric stretch and $NH_2$ symmetric stretch, respectively. At 3120 and 3180 $cm^{-1}$, two bands corresponding to $Csp^2$-H were recorded. The presence of a primary amine was also confirmed by means of a $NH_2$ deformation band at 1620 $cm^{-1}$ and finally by the presence of a very strong band between 860 and 760 $cm^{-1}$.

### 3.7. NMR

The relatively scarce information provided by MS for the structural elucidation made the use of NMR techniques necessary. The degradation product was fully characterized through the information obtained from $^1H$ and $^{13}C$ experiments.

The proton NMR spectrum of the degradation product, including an expansion of the aromatic region, is shown in Fig. 7. An inspection of the aromatic zone of the $^1H$ NMR spectrum revealed the presence of eight protons, five of which were justified by the intact unsubstituted phenyl ring, whereas the other three signals belong to the phenyl ring of the benzodiazepine structure.

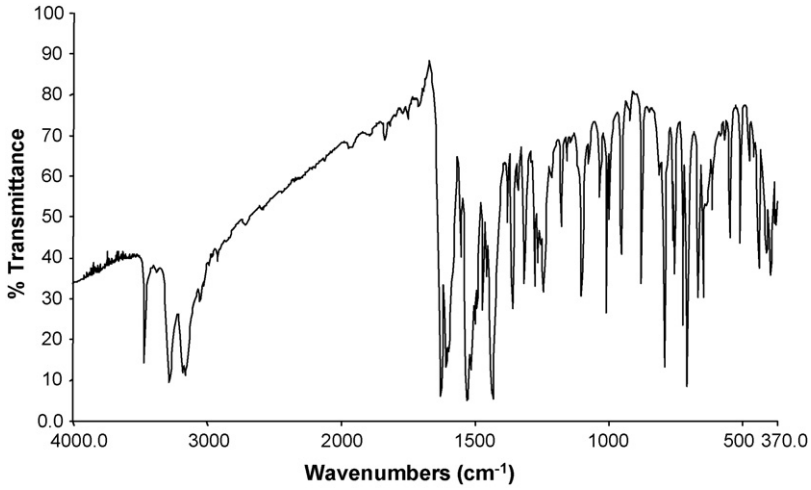


Fig. 6. Infrared transmission spectrum in KBr film at 1%.

Two signals appeared in the aliphatic region; the first one at 3.13 ppm was obviously assigned to one unique methyl group and the second which integrated for two protons at 4.74 ppm was interpreted as one primary amine. This last signal and the absence of two signals (4.0–5.5) corresponding to the pair of protons attached to C4 of the alprazolam structure suggested that the reversible 1,4-benzodiazepine ring opening had occurred.

The carbon spectrum and DEPT, as can be observed in Fig. 8, showed methyl presence, 8 Ternary carbons of which five correspond to the intact unsubstituted phenyl ring and the rest of the signals belonging to the phenyl ring of the benzodiazepine structure. The signal that appears at 112.39 ppm showed its quaternary carbon quality in DEPT experiment and this suggests that this atom might be attached to the primary amine group observed in the proton NMR spectrum. Finally, the absence of a secondary carbon in the structure corresponding to C4 of the alprazolam molecule confirms the ring-opening of the benzodiazepine system.

A detailed and complete assignation of the $^{1}$H NMR and $^{13}$C NMR signals is given in Table 2. The corresponding identity of this degradation product is 7-chloro-1-methyl-5-phenyl-[1,2,4]triazolo[4,3-a]quinolin-4-amine (triazolaminoquinoleine).

### 3.8. Synthesis and confirmation of the proposed structure

NMR results were quite similar to the spectrum reported by Nudelman et al., however the authors made clear that more than one structure was possible with those results. Therefore, in order

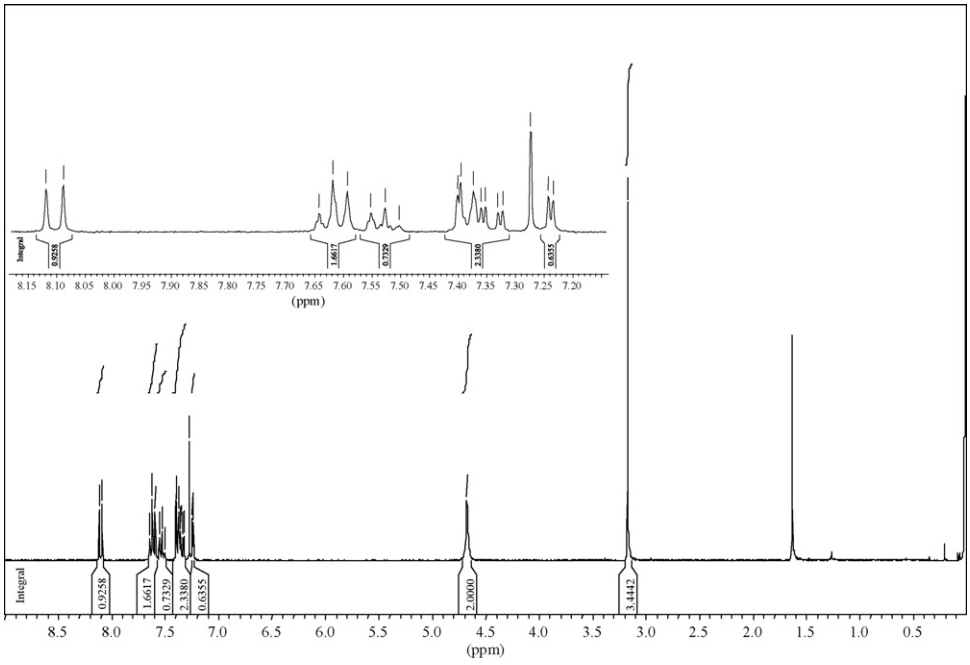


Fig. 7. Proton NMR spectrum of the isolated degradation product.

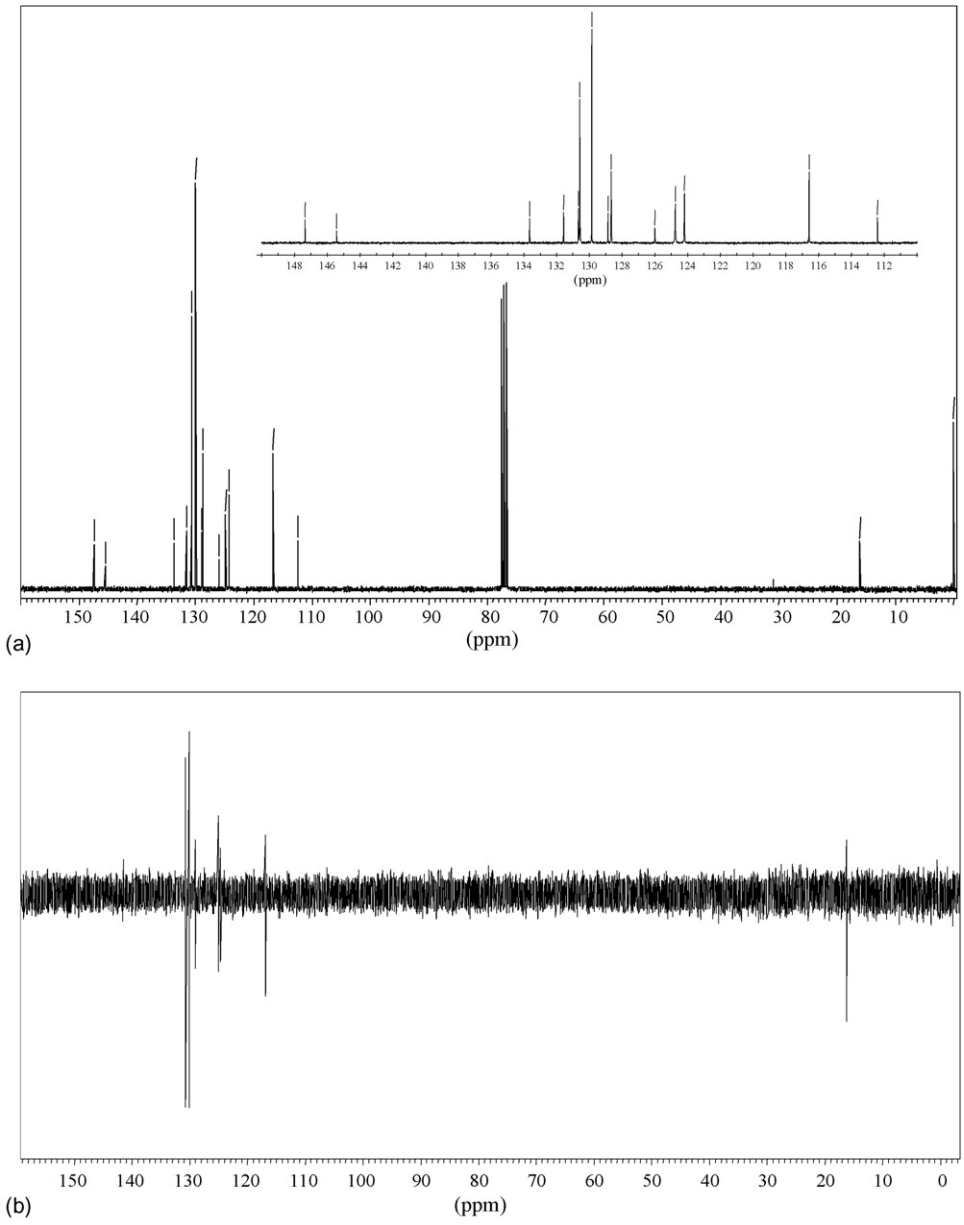


Fig. 8. (a) Carbon MNR spectrum and (b) DEPT spectrum of the isolated degradation product.

to achieve an unequivocal identification, triazolaminoquinoleine was synthesized in our laboratory following the method previously described [4].

The reaction yield was 94.3% and 180.2 mg of the reaction product were purified by recrystallization in 75 mL benzene at 50 °C. Finally, 153.3 mg of a white powder were obtained with a purity higher than 95% as determined by HPLC.

The synthesized compound was characterized by the same elucidation techniques employed for the isolated degradation product. UV (nm): $\lambda_{max}$ 195, 234, 315. IR ($cm^{-1}$): 3460, 3280, 1620. $^{1}H$ NMR ($CDCl_3$): $\delta$ (ppm): 3.13 (s, 3H); 4.74 (s, 2H); 7.20 (d, 1H, $J = 2.7$ Hz); 7.33 (dd, 1H, $J = 9.1$ Hz); 7.35 (d, 2H, $J = 6.6$ Hz); 7.49 (t, 1H, $J = 7.4$ Hz); 7.56 (t, 2H, $J = 7.4$ Hz); 8.07 (d, 1H, $J = 9.4$ Hz).$^{13}C$ NMR ($CDCl_3$): $\delta$ (ppm): 15.99 (1C); 112.84 (1C); 116.87 (1C); 124.51 (1C); 125.05 (1C); 126.21 (1C); 128.96 (1C); 129.16 (1C); 130.14 (2C); 130.84 (2C); 130.93 (1C); 131.93 (1C); 133.88 (1C); 145.44 (1C); 147.62 (1C). MS (*m*/*z*): 309. MS/MS (*m*/*z*): 267.9, 232.9.

Moreover, previous results of our laboratory showed that the impurity synthesized and the unknown degradation product exhibited the same retention time in the HPLC method and the same migration time in an orthogonal CE method developed for that purpose [8].

All results obtained by independent identification techniques demonstrate the correct assignation of the structure proposed here, as shown in Fig. 9, which agrees with a previous one described in literature, but is formed in very different conditions. In fact, in our study alprazolam API was stable to the most common degradation factors but its degradation, in tablets kept in blisters isolated from light, was very rapid with heat and

Table 2
NMR carbon and proton chemical shifts and DEPT results obtained for the degradation product

| $^{13}$C chemical shift (ppm) | DEPT | Integrated atoms | Assignment | $^{1}$H chemical shift (ppm) | Multiplicity | Integrated atoms | *J* (Hz) | Assignment |
|---|---|---|---|---|---|---|---|---|
| 15.99 | $CH_3$ | 1 | $CH_3$ at C1 | 3.13 | s | 3 | – | $CH_3$ at 1 |
| 112.39 | C | 1 | C-$NH_2$ at C4 | 4.74 | s | 2 | – | $NH_2$ at 4 |
| 116.57 | CH | 1 | C8 | 7.20 | d | 1 | 2.7 | H at 6 |
| 124.19 | C | 1 | C9a | 7.33 | dd | 1 | 9.1 | H at 8 |
| 124.74 | CH | 1 | C6 | 7.35 | d | 2 | 6.6 | H at 2′ and 6′ |
| 125.98 | CH | 1 | C9 | 7.49 | t | 1 | 7.4 | H at 4′ |
| 128.65 | C | 1 | C5 | 7.56 | t | 2 | 7.4 | H at 3′ and 5′ |
| 128.85 | C | 1 | C5a | 8.07 | d | 1 | 9.4 | H at 9 |
| 129.85 | CH | 2 | C2′ and C6′ | | | | | |
| 130.57 | CH | 2 | C3′ and C5′ | | | | | |
| 130.65 | CH | 1 | C4′ | | | | | |
| 131.55 | C | 1 | C7 | | | | | |
| 133.64 | C | 1 | C1′ | | | | | |
| 145.42 | C | 1 | C3a | | | | | |
| 147.35 | C | 1 | C1 | | | | | |

humidity, and it was completely dependent on the presence of excipients. Further studies will be necessary to determine the excipient or mixture responsible for the degradation in order to improve the stability of the formulation.

### 3.9. New HPLC method for alprazolam and triazolaminoquinoleine determination

To our knowledge, there is not a validated HPLC method including alprazolam and triazolaminoquinoleine determination described in the literature. Therefore, a method was developed for that purpose. In order to achive a lower retention for triazolaminoquinoleine different bonded phases were tested, such as $C_{18}$, $C_8$, RP-Amide. A Zorbax Eclipse XDB-$C_{18}$ 150 mm × 4.6 mm 5 μm; Zorbax (solvent saver) SB-Aq 150 mm × 3 mm 5 μm; Hypersil $C_{18}$ 200 mm × 4.6 mm 5 μm; Discovery $C_8$ 150 mm × 4.6 mm 5 μm; Discovery $C_{18}$ 150 mm × 4.6 mm 5 μm; Zorbax Eclipse Bonus RP 150 mm × 4.6 mm 5 μm; Ascentis RP-Amide 150 mm × 4.6 mm 5 μm and Ascentis $C_{18}$ 150 mm × 4.6 mm 5 μm columns were tested following such strategy. Shorter analysis time was obtained with a $C_8$ column in such a way that no differences in $k'$ for alprazolam were found while triazolaminoquinoleine was retained less.

Using the conditions described above in Section 2.11, it was possible to separate the alprazolam and its impurity in less than 3 min. Fig. 10 shows the chromatogram obtained for the excipients and the sample under these conditions. It must be pointed that the sample analyzed is the same as the one in the Fig. 1.

Validation was performed following ICH guidelines [9–12] with standards and a synthetic mixture of alprazolam tablet corresponding to a 0.25 mg dose. Validation parameters are summarized in Table 3.

Standards showed a good linearity as much for alprazolam in the quantification range as for triazolaminoquinoleine in the impurities range, with correlation coefficients over 0.999. A small bias was found in the regression line for both analytes, because it did not include the zero value. It could be mostly

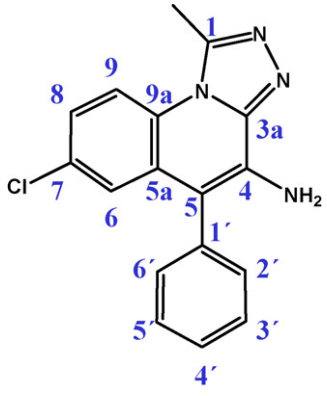


Fig. 9. Elucidated structure for the degradation product. Atom numbering refers to NMR assignments collected in Table 1.

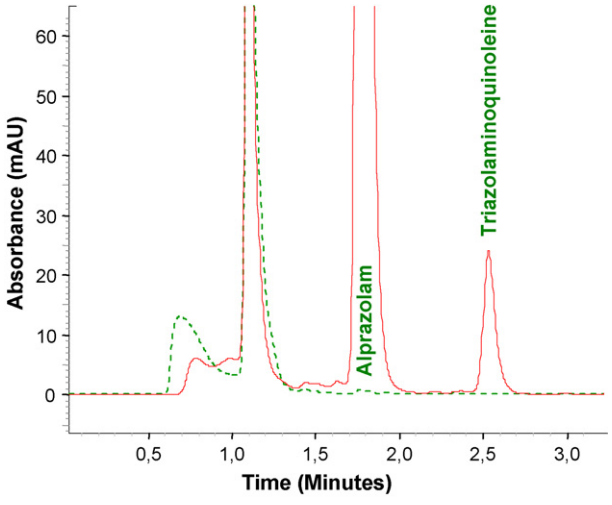


Fig. 10. Chromatograms corresponding to: tablet of alprazolam 0.25 mg dose containing triazolaminoquinoleine (continuous line); Placebo (discontinuous lines). Conditions: Discovery $C_8$ column, 5 μm particle size, 150 × 4.6 mm kept at 40 °C. The mobile phase consisted of phosphate buffer 25 mM (pH 7.0)/acetonitrile 43:57 (v/v). Flow rate, 1.5 mL/min, UV detection 234 nm and injection volume 20 μL.

Table 3
Main validation parameters for alprazolam and triazolaminoquinoleine

| | Alprazolam (quantification range) | Triazolaminoquinoleine (impurities range) |
|---|---|---|
| Standards linearity | | |
| Range ($\mu g\,mL^{-1}$) | 30.5–50.8 | 0.02–2.10 |
| Intercept ± C.L. | 222570 ± 212937 | 587 ± 832 |
| Slope ± C.L. | 220302 ± 5165 | 374130 ± 864 |
| $r$ | 0.9992 | 1.0000 |
| Sample linearity | | |
| Range ($\mu g\,mL^{-1}$) | 32.5–48.8 | 0.02–2.10 |
| Intercept ± C.L. | −208738 ± 165793 | 186 ± 1113 |
| Slope ± C.L. | 231273 ± 2325 | 370451 ± 1156 |
| $r$ | 0.995 | 1.0000 |
| Accuracy (% recovery) | | |
| Standard | 100.01 | 99.03 |
| R.S.D. (%) | 0.70 | 2.92 |
| Sample | 100.04 | 98.90 |
| R.S.D. (%) | 1.52 | 2.34 |
| Standards precision instrumental | | |
| Intra-assay ($n = 10$) | | |
| Mean (μg/mL) | 40.6 | 0.21 |
| R.S.D. (%) | 0.15 | 0.48 |
| Intermediate ($n = 20$) | | |
| Mean (μg/mL) | 40.6 | 0.20 |
| R.S.D. (%) | 0.63 | 0.79 |
| Standards precision method | | |
| Intra-assay ($n = 10$) | | |
| Mean (μg/mL) | 40.6 | 0.21 |
| R.S.D. (%) | 0.96 | 0.79 |
| Intermediate ($n = 20$) | | |
| Mean (μg/mL) | 40.6 | 0.20 |
| R.S.D. (%) | 1.04 | 2.01 |
| Samples precision method | | |
| Intra-assay ($n = 10$) | | |
| Mean (μg/mL) | 40.6 | 0.21 |
| R.S.D. (%) | 1.15 | 0.79 |
| Intermediate ($n = 20$) | | |
| Mean (μg/mL) | 40.7 | 0.20 |
| R.S.D. (%) | 1.34 | 0.95 |

justified by the good fit of the points to the regression lines, which makes the limits of confidence for the intercept very narrow, and it had no practical effect. Recoveries did not statistically differ from 100% ($t$-test, $p < 0.05$) in any case. R.S.D. values were low enough to consider the method precise as much for standards as for alprazolam tablet samples. Limits of detection and quantification for Triazolaminoquinoleine were 0.02% and 0.005%, respectively. The real limit of quantification of one method is the lower concentration value where it has been validated and therefore, it is 0.05%. Limits of detection were under the necessary values for the method.

## 4. Conclusions

An efficient isolation method, a set of elucidation techniques, as well as the synthesis of the compound have been successfully applied to the identification of a degradation product that appears in alprazolam tablets. Accelerated degradation studies have proven that this impurity, identified as triazolaminoquinoleine, is generated from the interaction between alprazolam and excipients under high temperature and humidity conditions, but is independent of the presence of light.

A rapid HPLC method with UV detection has been developed that allows the simultaneous determination of alprazolam and triazolaminoquinoleine in less than 3 min of total run. This method has been properly validated and it has been shown that it is reliable, being linear, accurate and precise.

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