Identification of Alprazolam and its degradation products using LC-MS-MS

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This paper is dedicated to Professor António-Rocha Gonsalves on the Occasion of his 70th Birthday. This work is a tribute to and also the result of his lifelong collaboration with several pharmaceutical companies in Portugal and abroad and his pioneering role in establishing the use of HPLC, MS and later LC-MS-MS both at some of those companies and at several research institutions, to study real and complex chemical problems.

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Abstract

An efficient LC/MS/MS methodology for the analysis of Alprazolam API and Alprazolam tablets was developed. The analysis of the MS^2 fragmentation profile, obtained from only one chromatographic assay, allows the identification of Alprazolam, its main degradation product, triazoaminoquinoleine (Imp G), the analysis of the MS^3 spectra allows the identification of 5-chloro-[5-methyl-4*H*-1,2,4-triazol-4-yl]benzophenone (Imp C) and with SIM mode the 5-chloro-[2-(3-aminomethyl-5-methyl-1,2,4-triazol-4-yl]benzophenone, opened-ring Alprazolam, proposed as precursor of triazoaminoquinoleine in basic and acid hydrolysis and only detected before by NMR analysis, was identified. With this method it is possible to study samples with only one chromatographic run, identifying unequivocally the impurities present, taking advantage of the MS-MS resource.

Keywords: Alprazolam, LC-MS-MS, forced degradation, degradation products

Introduction

Alprazolam (AL) is a triazolobenzodiazepine used extensively for the control of panic attacks and in the management of anxiety disorders.[1] The concern of the pharmaceutical industry with the quality of the product, in particular with the percentage of known and unknown impurities in the commercial formulation during the life time of the drug, stimulated the commencement of studies for the identification and quantification of the impurities of AL formulations. The stability of AL has been studied with respect to the photostability and excipient influence using LC-MS,[2] spectrofluorimetric assay,[3] capillary electrophoresis[4] and HPLC.[5-7]

Drug degradation in formulations is a very complex process that can be influenced by the structure of the API, the composition of the tablets, the formulation process and the storage conditions including temperature, humidity, light and containers. LC-MS-MS is a very powerful technique for the analysis of low-level degradation products without the need for a laborious isolation processes.

In 2002 Nudelman and Cabrera found that the drug is sensitive to both artificial light and to sunlight, the main degradation products being triazolaminoquinoleine, 5-chloro-[5-methyl-4*H*-1,2,4-triazol-4-yl]benzophenone (registered respectively as Imp G and Imp C in the Pharmacopoeia) and 1-methyl-6-phenyl-4*H*-s-triazolo[4,3-*a*]-1,4-benzodiazepinone.[8] In 2005, the same group reported studies of the hydrolysis and photodegradation of Alprazolam, emphasizing that Imp G is the result of a photodegradation. The authors suggested that the ring opening is a reversible reaction and they detected the presence of the opened-ring Alprazolam in the reaction mixture only by NMR analysis.[9] The different stability of AL against hydrolysis when it is in the presence of different excipients was studied concluding that Imp G is formed from the reaction of opened-ring Alprazolam with magnesium stearate and carboxymethyl cellulose (CMC).[6] Barbas *et al.*, in 2007, verifed that Imp G, is formed rapidly in the presence of excipients under high temperature and humidity conditions, independently of the presence of light. An LC method for the determination of AL and Imp G was developed and validated but no fragmentation profile was achieved in MS.[2] Studying the influence of the different excipients, the authors conclude that Alprazolam degradation might be mediated by the Maillard reaction, including ring opening of Alprazolam and lactose leading to Imp G.[10]

In this paper a LC-MS-MS method for the determination of AL and related impurities, Structure Blocks 1, is described and the fragmentation profile of degradation compounds was obtained, allowing for their unequivocal identification. Degradation studies of API were performed in order to obtain a higher level of impurities allowing their identification with the developed method. The methodology developed also allows the detection of opened-ring Alprazolam postulated as the precursor of the Alprazolam degradation in acid and basic hydrolysis and undetectable with the other methodologies used to analyze Alprazolam and Alprazolam tablets.

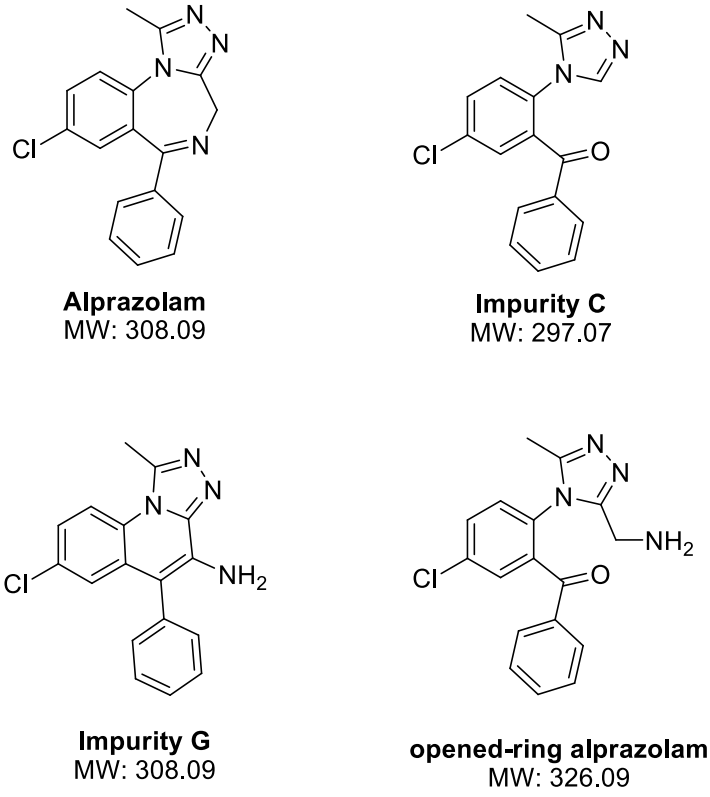


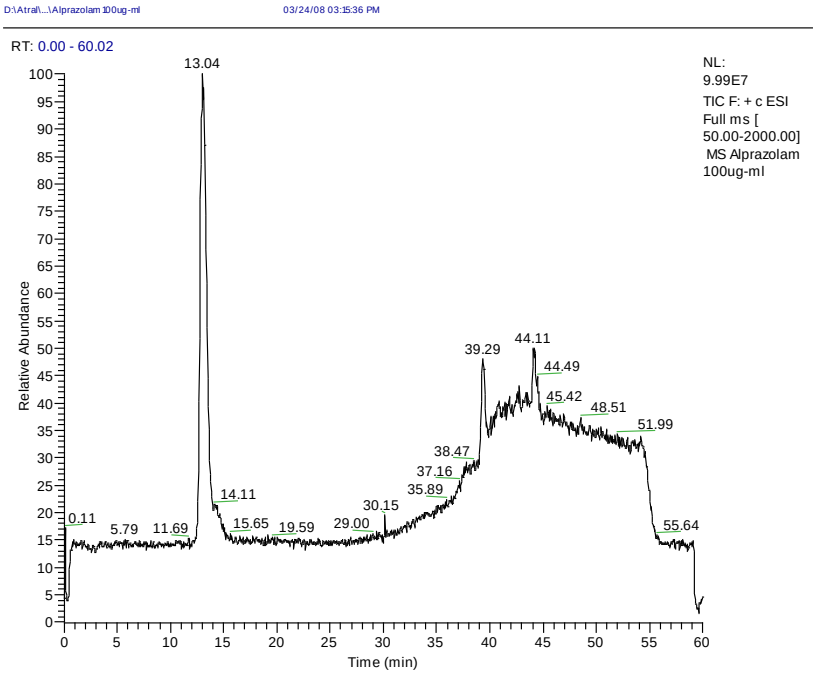
Chart 1. Structures and molecular weights of Alprazolam and related impurities.

Result and Discussion

Chromatographic profile of Alprazolam tablets and identification of impurities

Commercial brands (CB) of Alprazolam in dosages of 0.25 mg, 0.50 mg and 1.0 mg, and API, were analyzed using LC-MS-MS. Figure 1 shows the LC chromatogram of commercial brand and API standard using solutions of 0.1 mg/mL. MS confirmation of the analytes was always performed when small differences of retention times were observed from experiments performed on different days. The relative areas from the MS of each chromatographic peak, and the corresponding $(M+1)^+$ values from the API chromatogram and from several commercial brands, with different dosages and expiration date are presented in Table 1.

a)



b)

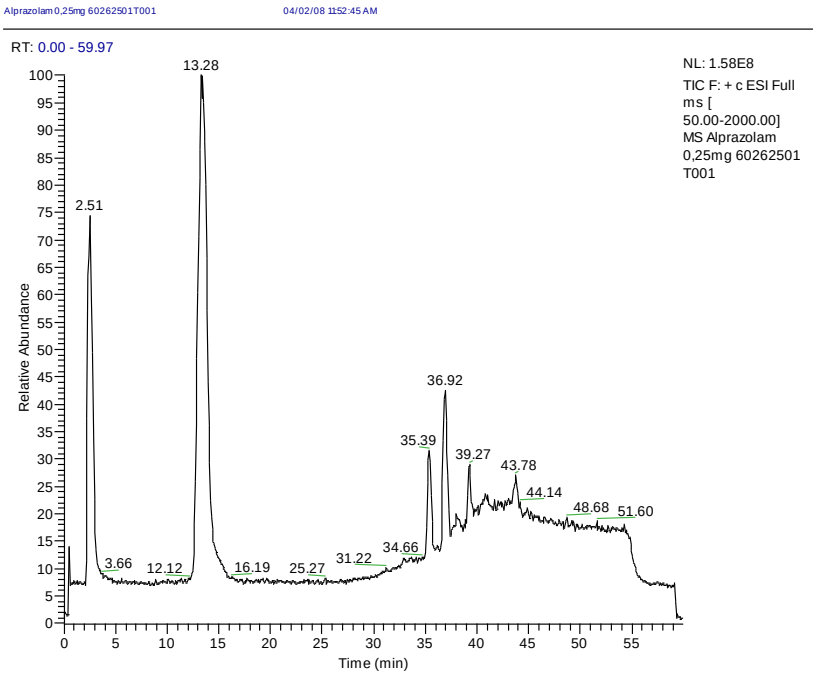


Figure 1. LC chromatogram of AL (a) and Commercial Brand (b).

Table 1. Characteristic chromatographic profile of Alprazolam (API) and commercial brands

Name	Dosage (mg)	Expiration date	Area relative (%) Retention time (min) , $(M+1)^+$				
			2.51min, 343	13.4min, 309	34.3 309	35.4 undefined	36.9, undefined
API standard		07/2010	--	100	--	--	--
CB-1	0.25	01/2011	25.04	61.38	--	5.32	8.26
CB-2	0.5	11/2010	25.02	60.9	--	6.39	7.68
CB-3	1.0	11/2010	22.14	66.02	--	4.85	6.99
CB-4	0.25	10/2007	17.95	63.64	1.62	7.35	9.44
CB-5	0.5	12/2007	24.51	62.93	1.23	4.02	7.31
CB-6	1.0	10/2007	19.90	69.82	0.52	3.76	6.00

At 13.4 minutes the characteristic chromatographic peak of AL was observed with $(M+1)^+$ of 309. The peak with retention time 2.51 min and $(M+1)^+$ of 343 was identified as lactose. All tablets present two small peaks at 35.4 min and 36.9 min that we could not identify through the MS spectra. In the chromatogram of the 1.0 mg dosage, a peak with retention time of 34.3 min and $(M+1)^+$ of 309 was observable.

Alprazolam tablets were previously analyzed by LC-MS using an ODS Hypersil column and ammonium acetate 25 mM (pH 4.2):acetonitrile (45:55) as isocratic mobile phase but under these conditions the fragmentation profile of chromatographic peaks with *m/z* 309 was not obtained making the differentiation of these two peaks impossible.[2] Using the LC-MS-MS method developed by us it is possible to achieve the MS^2 of each peak, obtaining the fragmentation profile that allows the unequivocal identification of both peaks, Figure 2. The MS^2 analysis of the peak at 13.4 min presents the following main fragments: 309 (M+1), 281 (100), 274 and 205. The more abundant peak of AL, the fragmentation peak at *m/z* 281, could be interpreted as the loss of N_2, the peak at *m/z* 274 is formed from the cleavage of the C-Cl bond (confirmed by the isotopic analysis of ESI/MS-MS experiments) and the peak at *m/z* 205 could be the 4-(4-chlorophenyl)-4*H*-[1,2,4]triazole ring. We assigned this peak to AL, also in agreement with the analysis of API. The peak at 34.3 min presents in the MS^2 analysis, fragments at 309 (M+1), 268 (100) and 233 (Figure 2); we assigned this chromatographic peak to triazolaminoquinoleine.[11] The peak at *m/z* 268 was interpreted as a possible elimination of acetonitrile and the peak at *m/z* 233 as a loss of a chlorine atom from the fragment at *m/z* 268 (confirmed by isotopic analysis in ESI/MS-MS experiments). Figure 2 shows the MS^2 analysis of both peaks and the assignment of the main fragments. The broad band obtained for alprazolam is due to the high concentration (100 μg/ml) required to see the impurity and to carry out the MS^2 experiment on the impurity.

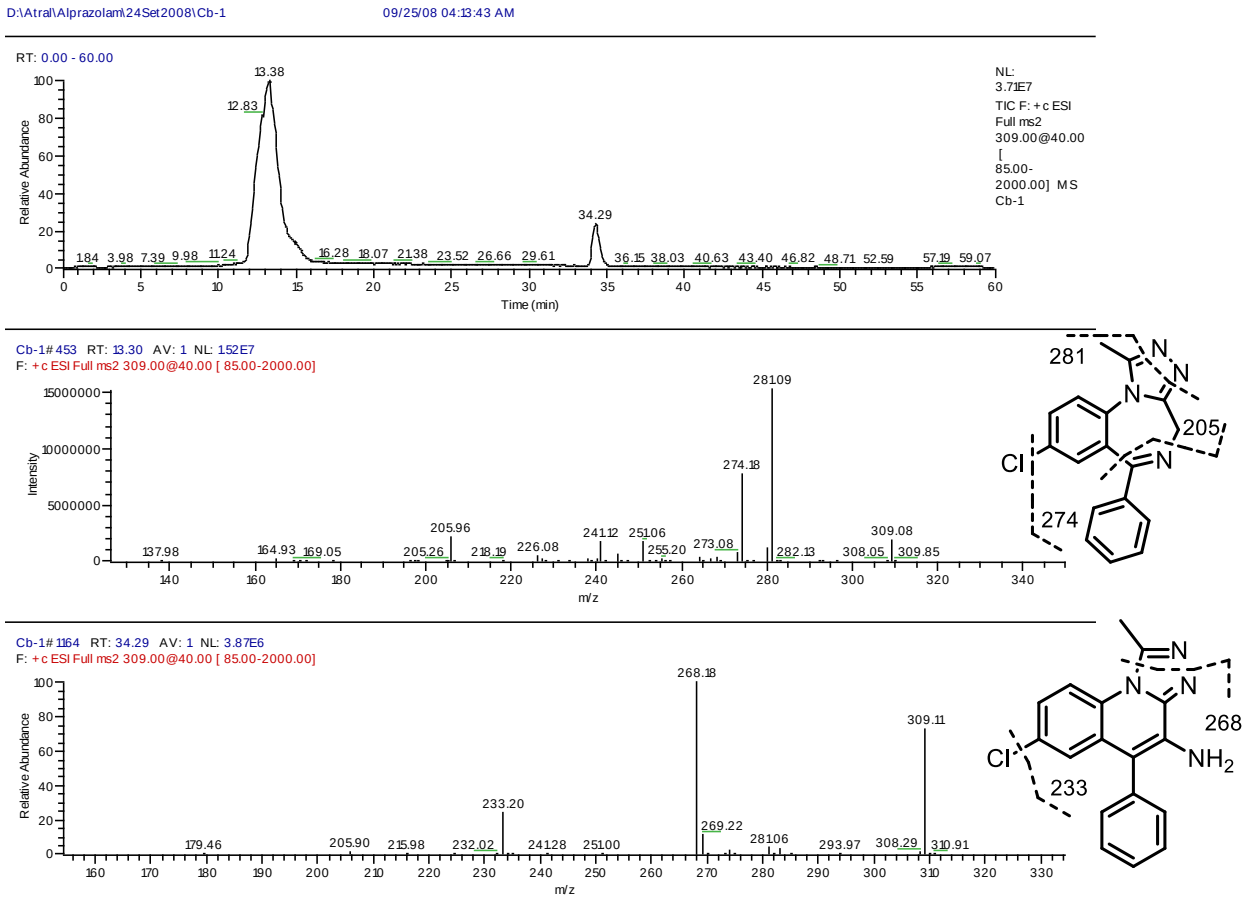


Figure 2. MS^2 analysis of the chromatographic peaks with *m/z* 309.

In order to validate our assignment we synthesized Imp G, following the methodology described by Nudelman *et al.*[8] – acid hydrolysis of Alprazolam with HCl, 0.33M in methanol, followed by cyclization in refluxing methanol, with KOH 2.0 N. Instead of extraction with benzene and chromatographic separation, the isolation was achieved after neutralization with HCl 1N by extraction of triazolaminoquinoleine with methylene chloride. After recrystallization from methylene chloride, triazolaminoquinoleine was characterized by ^{1}H-NMR, ^{13}C-NMR, elemental analysis, FTIR and UV-VIS spectroscopy. Figure 3 shows the UV-VIS spectra of AL and Imp G. The absorption coefficient (ε) was determined in mobile phase A and B at 254 nm for AL and for Imp G the absorption coefficient at the maxima of the absorption bands was calculated in both solvents, Table 2. Considering the differences in the absorption coefficients in the two mobile phases used, the areas measured by LC do not represent the ratio between AL and Imp G; the concentration of Imp G corresponds to *ca.* half of the area measured.

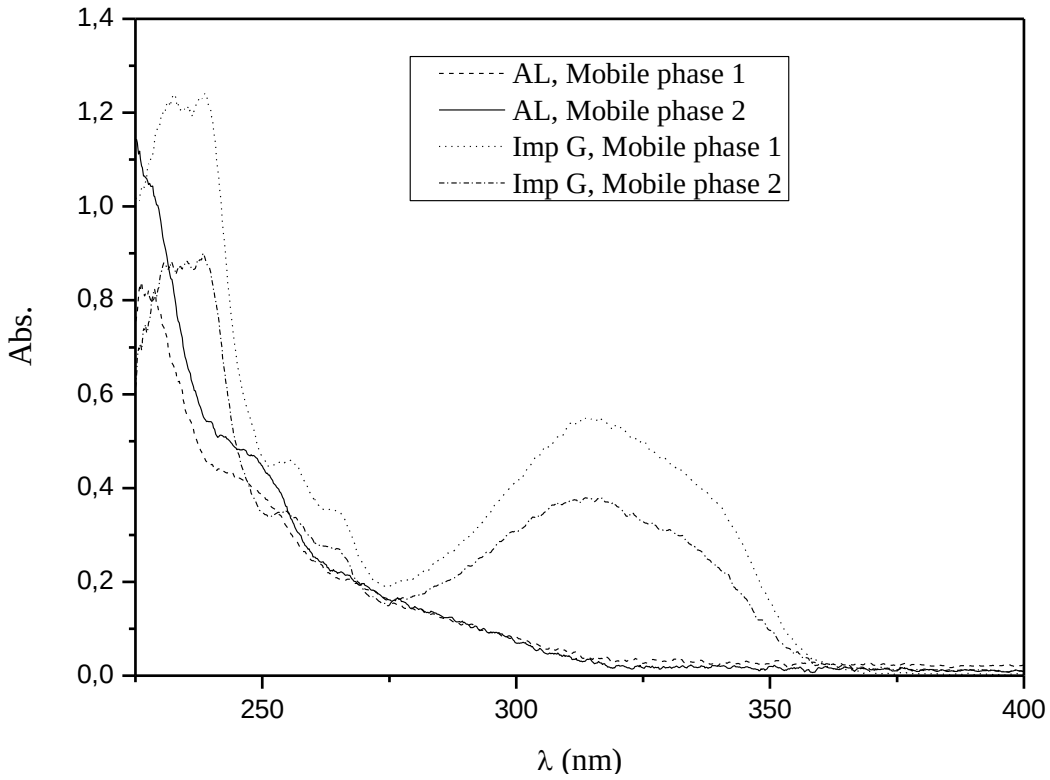


Figure 3. UV-VIS spectra of Alprazolam and Imp G in mobile phase 1 and mobile phase 2.

Table 2. Maximum of absorption bands and absorption coefficients of Alprazolam in the two mobile phases used in the LC-MS-MS method

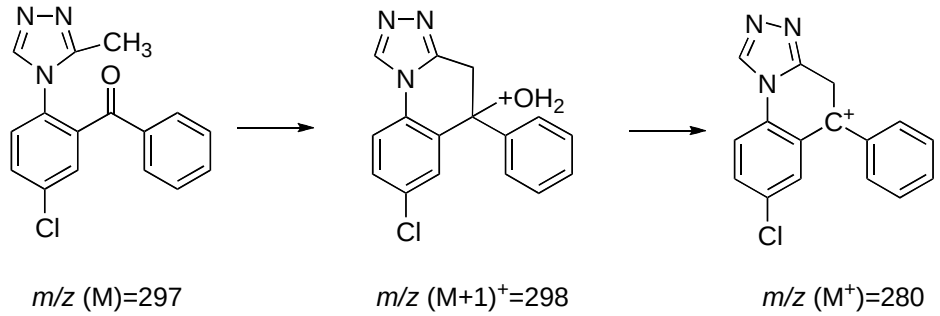
Compound	λ (nm) ε $(mg/mL)^{-1}cm^{-1}$		
AL Mobile phase A	254 30.75		
AL Mobile phase B	254 39.82		
Imp G Mobile phase A	245 34.36	263 26.66	315 38.48
Imp G Mobile phase B	254 61.19	263 48.39	315 74.56

The LC-MS-MS analysis of triazolaminoquinoleine shows only one peak at 34.2 minutes with the same MS^2 fragmentation pattern that was obtained in the analysis of Alprazolam tablets.

Alprazolam and Imp G were quantified. Standards of alprazolam showed a good linearity with correlation coefficient of 0.993 and $y = 2x10^7x+8x10^8$ as calibration curve. The limits of quantification and detection obtained were 50 μg/ml (%RSD = 7.38) and 1 μg/ml, respectively. Standards of Imp G showed a good linearity with correlation coefficient of 0.997 and $y = 3x10^7x+5x10^7$ as calibration curve. The limits of quantification and detection obtained were 0.05 μg/ml (%RSD = 3.87) and 0.025 μg/ml, respectively.

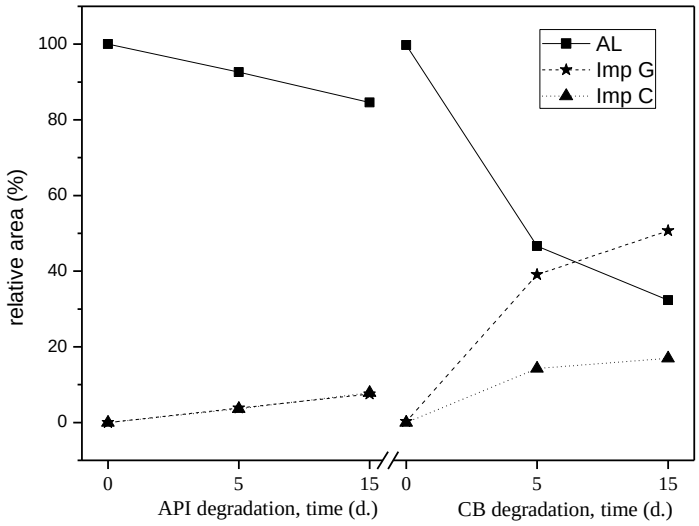
Forced conditions

High humidity and temperature are factors known to be responsible for an increase in the rate of degradation of AL.[2] In order to probe the utility of the LC-MS-MS method to detect other known impurities, degradation of AL and commercial brands (CB) was accelerated. Alprazolam API and tablets of 0.25 mg (expiration date 01/2011) were dissolved in equal parts of methanol/citrate buffer and kept in a water bath at 50 ºC in open vessels for 15 days. Aliquots were analysed during this period of time. In the LC chromatograms of AL and commercial tablets it was possible to identify, after 3 days, Imp C at 9.89 min.[11] The MS spectrum showed an ion at *m/z* 298 corresponding to $(M+1)^+$. The MS^2 spectrum showed one peak only at *m/z* 280, in order to obtain a fragmentation profile we performed a MS^3 spectrum, Figure 4. The main fragment is most likely to come from a water elimination originating from cyclization as proposed in Scheme 2.



Scheme 2. Proposal for the formation of the main fragment of impurity C.

Graph 1 shows the evolution of the relative percentage of Alprazolam, Imp C and Imp G in the forced degradation conditions. The degradation rate is higher in the CB than in the API solutions pointing out the influence of the excipients in this process. After 15 days the samples of AL present 7.54 % of Imp G and in the samples of commercial tablets the percentage of Imp G is 50.65, almost seven times higher than that obtained in the absence of excipients.



Graph 1. Evolution of the relative percentage of Alprazolam, Imp C and Imp G in the forced degradation conditions

The opened-ring Alprazolam is the product of hydrolysis of Alprazolam. Hydrolytic breakdown leading to a benzophenone derivative has also been described for other benzodiazepines such as triazolam[12] or loprazolam[13] as well as for the thienotriazolodiazepine known as brotizolam,[14] as a room temperature reaction. The opened-ring Alprazolam is described for Barbas[10] and Nudelman and Cabrera[9] as the precursor of the degradation reactions, involving different excipients, leading to the formation of Imp G, but this compound was only detectable by NMR analysis.

In order to test our developed methodology we performed a forced degradation of AL with HCl 1M and MeOH:HCl 1M (1:1). The LC-MS-MS analysis of AL dissolved in HCl showed a small peak at 5.85 min. A peak with the same retention time, but more intense, was obtained using MeOH:HCl, Figure 4. Both solutions were heated in reflux overnight, when an increase of the area of the peak was observed. The MS^2 analysis of this peak presented the following main fragments: 327 (M+1), 310 and 298 (100), Figure 5. The fragmentation peak at *m/z* 310, could be interpreted as the loss of NH_3, the peak at *m/z* 298 is formed from the loss of CH_2NH_3. We confirmed, by isotopic analysis of ESI/MS-MS experiments, that the ion at *m/e* 329 gives a fragmentation pattern 329 (M+1) $\rightarrow$ 312 $\rightarrow$ 300 (100). The spectrum of this compound has the M+1 of the opened-ring Alprazolam and the fragmentation profile is consistent with that structure.

Using the SIM mode it was possible to detect this compound in the chromatograms of the tablets submit to the forced degradation conditions described above using MeOH:citrate buffer solutions heated at 50 ºC for 15 days.

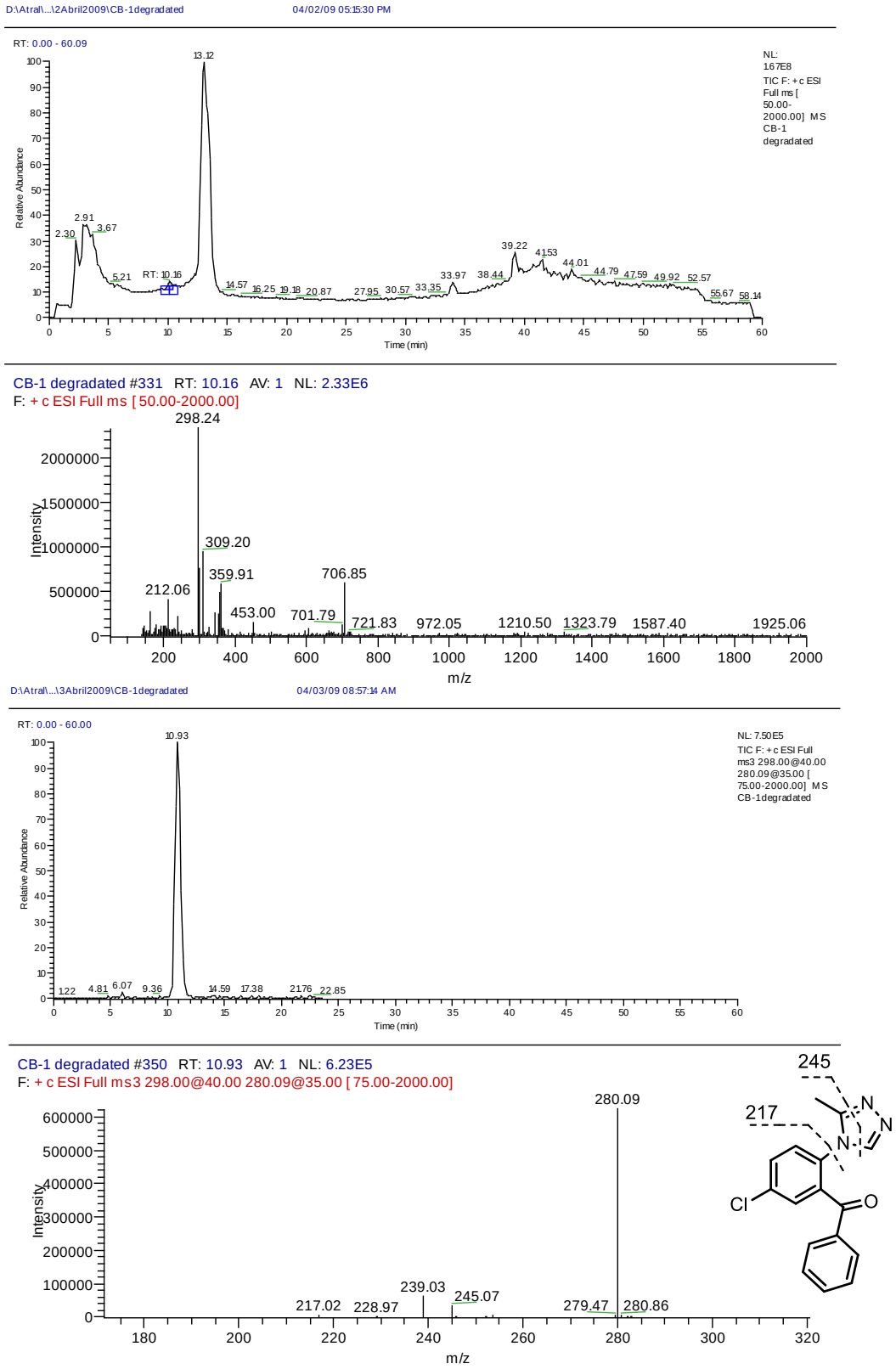


Figure 4. Idenfication and MS^3 spectra of Impurity C.

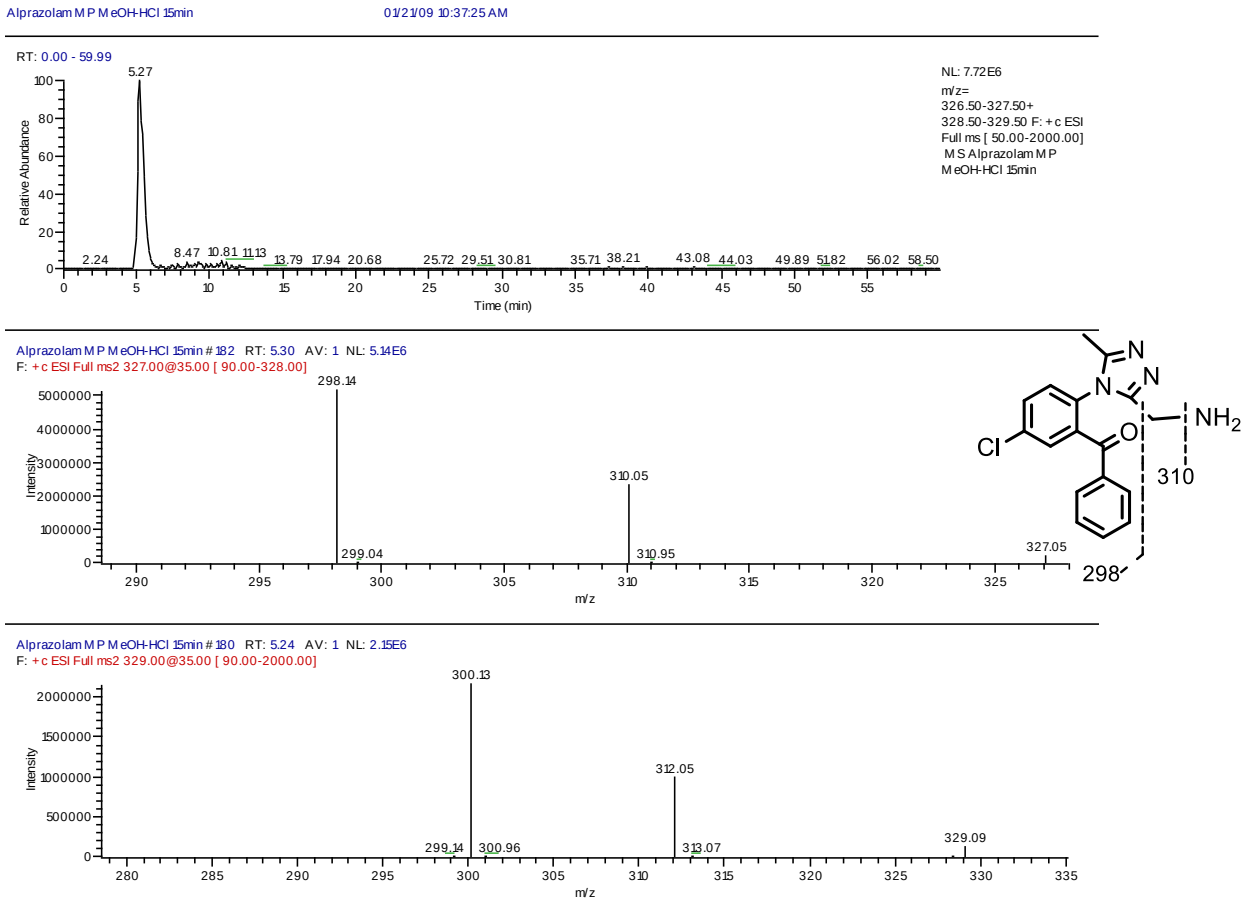


Figure 5. Fragmentation of opened-ring Alprazolam.

Conclusions

The LC-MS-MS methodology developed for the analysis of Alprazolam API and commercial brands allows the separation and identification of Alprazolam and related impurities, impurity C and impurity G, as well as the product of the hydrolysis reaction in acid media, the opened-ring Alprazolam, in just one chromatographic experiment. This involves the identification of impurity G throught MS^2 analysis and detection of opened-ring Alprazolam in SIM mode in one chromatographic assay.

Experimental Section

General. The standard of Alprazolam and tablets of Alprazolam (1.0, 0.5 and 0.25 mg) are over the counter pharmaceuticals. Methanol Chromasolv was obrtained from Sigma-Aldrich,

ammonium acetate pro analysis and hydrogen chloride analytical from Riedel de Haën, glacial acetic acid pro analysis from Merck, sodium chloride (current laboratory brand), potassium hydroxide EKA pellets from Akzo Nobel, acetic anhydride pro analysis from Panreac. Tetrahydrofuran and methylene chloride were dried and distilled prior to use following standard procedures and water was purified with a Milli-Q plus system from Millipore. Alprazolam and synthesized triazoaminoquinoleine, were analyzed by Fourier infrared spectroscopy with a FTIR spectrometer (Thermo Nicolet 6700) from 4000 to 400 cm^{-1}. The samples were dispersed in KBr pellets. NMR spectra of Alprazolam standard and triazoaminoquinoleine were acquired on an NMR BRUKER Avance III 300 MHz spectrometer and in MeOD at 298 K. Elemental analysis of the Alprazolam standard and of synthesized triazoaminoquinoleine were performed on a Fisons Instrument EA 1108 CHNS-O.

Methods

Sample preparation

Alprazolam and triazoaminoquinoleine reference solutions were prepared by accurately weighing samples and diluting in methanol to obtain solutions of 100 μg/ml of these compounds, and studies of direct infusion in mass spectrometry and in LC-MS-MS were performed. The tablets of 1.0 mg, 0.5 mg and 0.25 mg were triturated and weighted in order to obtain solutions of 100 μg/ml of Alprazolam in methanol. Before injection in LC-MS-MS, these samples were placed in an ultra-sound bath for 15 min then centrifuged for 10 min.

LC-MS-MS

LC-MS-MS experiments were performed on a LC Surveyor system with a photo diode array detector coupled to an ion trap Finnigan Advantage mass spectrometer equipped with an ESI source. Liquid chromatography (20 μl injection) was performed with a Synergi Phenomenex Polar RP column 150 mm× 2.0 mm, 4 μm fitted with a guard column. The guard and analytical columns were kept at 40 ºC and the mobile phase A was ammonium acetate buffer (pH 4.2; 0.1M)/methanol, 44/56 (v/v) and mobile phase B was ammonium acetate buffer (pH 4.2; 0.1M)/methanol, 5/95 (v/v). The flow rate of the mobile phase was maintained at 200 μl/min. Good chromatographic separation of Alprazolam and related impurities was achieved using the following optimized linear gradient elution: 0-20 min, 98% of mobile phase A, 20-35 min, linear gradient decrease to 1% of mobile phase A, maintained until 50 min, 50-51 min, linear gradient increase of mobile phase A to 98% and finally these proportions maintained for 60 min. UV detection was performed at 254 nm and spectral data was collected in the chromatographic run between 200-600 nm.

The MS parameters were positive polarity and capillary temperature 200 ºC. The MS optimized parameters were achieved acquiring Alprazolam solutions in direct infusion. The following tune parameters were used during analysis of samples: ESI voltage 4.5 kV; sheath gas flow, 60 (arbitrary units); auxiliary gas flow, 20 (arbitrary units); capillary voltage, 3.0 V; tube lens offset,

55 V. MS detection was performed in two scan events: total ion current (TIC) and Full MS^2 of M+1=309, M+1= 298 and M+1= 327 with collision energy 40% and isolation width (*m/z*) = 1.0.

Quantification studies of Alprazolam and major impurity

Selectivity was studied by running samples with the excipients used in formulations to show that there were no peaks at the same retention times of the studied analites.

Quantification parameters were tested for Alprazolam and Imp G in two different ranges. For Alprazolam, the linearity was studied in the range 50-120 μg/ml analyzing triplicate standard solutions at five concentration levels. For Imp G, the linearity was determined at the range of 0.05-5% (0.05-5 μg/ml) of impurity to API. Al tablet samples were prepared with 100 μg/ml of Alprazolam. The limit of quantification for Alprazolam and Imp G was determined by validating the lower level of each range. The limit of detection was calculated as 3x signal-to-noise ratio (3*S/N) and checked experimentally.

UV-VIS

Absorption spectra were recorded with a Shimadzu UV-2100 machine. Solutions of AL and triazoaminoquinoleine in ammonium acetate buffer (pH 4.2; 0.1M)/methanol, 44/56 (v/v) (mobile phase A) and ammonium acetate buffer (pH 4.2; 0.1M)/methanol, 5/95 (v/v) (mobile phase B) with concentrations between 0.03 mg/mL(*ca*. 0.1 mM) and 0.001 mg/mL (*ca*. 0.003mM) were measured. The absorption coefficient at 254 nm was calculated and confirmed the operation of the Beer-Lambert law in this interval. For the synthesized triazoaminoquinoleine the absorption coefficient was calculated for the maxima of absorption of each band at 254, 263 and 315 nm.

Triazoaminoquinoleine synthesis

Triazoaminoquinoleine was synthesized adapting the methodology described by Nudelman *et al.*,[8] with some modifications in the compound isolation. The reaction mixture was neutralized with 1N HCl. The solution was extracted with methylene chloride and the organic phase was evaporated. The extract was purified by recristallization from methylene chloride. The overall yield was 89%. ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 8.31 (dd, 1H, *J* = 9 Hz, *J* = 3Hz); 7.68-7.56 (m, 3H); 7.44-7.80 (m, 3H); 7.11 (t, 1H, *J* = 2.4 Hz); 3.16 (s, 3H). Elemental Analysis: Calc for $C_{17}H_{13}ClN_4$ C, 66.13; H, 4.24; N, 18.15%; found C, 65.53, H, 4.05; N, 18.13% UV-VIS (ammonium acetate buffer (pH 4.2; 0.1M)/methanol, 44/56 (v/v)) λ(nm), ε ($(mg/mL)^{-1}cm^{-1}$) 254, 34.36; 263, 26.66; 315, 38.48. UV-VIS(ammonium acetate buffer (pH 4.2; 0.1M)/methanol, 5/95 (v/v)) λ(nm), ε ($(mg/mL)^{-1}cm^{-1}$) 254, 61.19; 263, 48.39; 315, 74.56. FTIR (KBr) ν (cm^{-1}) 3160-3475 stretch, 1600-1630 bending, 700-800 wag of primary amine.

Degradation conditions

Tablets of 0.25 mg were triturated and dissolved with the same volume of methanol and citrate buffer (pH=2.0). These samples were maintained in a water bath at 50 ºC unprotected from light,

to obtain excess humidity and light conditions, during 15 days. The samples were analyzed at immediately after preparation and during 15 days.
The hydrolysis of Alprazolam was performed dissolving the API in HCl 2M and in a MeOH:HCl (1:1) mixture.

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