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Development and validation of a stability-indicating HPLC-UV method for the determination of alizapride and its degradation products

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ABSTRACT

A stability-indicating high-performance liquid chromatography procedure has been developed for the determination of alizapride (AL) and its main degradation products alizapride carboxylic acid (AL-CA) and alizapride *N*-oxide (AL-NO2) in drug substance and product. The method was developed based on forced degradation data obtained by HPLC–MS analysis. Indeed AL underwent chemical degradation by acid/base catalyzed hydrolysis and oxidation the main degradation products being AL-CA and AL-NO2 respectively. The separation and quantisation were achieved on a 150-mm reverse phase column with a hydrophilic linkage between silica particles and hydrophobic alkyl chains. The mobile phase was constituted (flow rate 1.5 mL min^{-1}) of eluant A: aqueous acetate buffer (pH 4.0; 20 mM) and eluant B: CH_3OH using a gradient elution and detection of analytes at 225 nm. The method showed good linearity for the AL, AL-CA, AL-NO2 mixture in the 25–75, 1–15 and 1–15 μg mL^{-1} ranges respectively, being all the square of the correlation coefficients greater than 0.999. The precision, determined in terms of intra-day and inter-day precisions and expressed as RSDs were 0.8, 1.3 and 2.1% and 1.0, 1.7, 4.8% for AL, AL-CA and AL-NO2 respectively. The method demonstrated also to be accurate; indeed the average recoveries, at 100% and 0.2% of the target assay concentration, were 100.5, 98.6, and 96.8% for AL, AL-CA and AL-NO2 respectively. The robustness was also evaluated by variations of mobile phase composition and pH. Finally, the applicability of the method was evaluated in commercial dosage form analysis as well as in stability studies.

1. Introduction

Alizapride, (±)-*N*-[(1-allyl-2-pyrrolidinyl)methyl]-6-methoxy-*1H*-benzotriazole-5-carboxamide (AL) (Fig. 1) is a substituted benzamide available as hydrochloride with potent anti-emetic properties. It is currently employed for the prevention of cancer chemotherapy and postoperative side-effects like nausea and vomiting and it is commercially available as injectable solution and tablets [1,2]. Several HPLC methods have been reported in literature for AL analysis but they are mainly related to its determination in biological fluids [3–7]. On the contrary, to the best of our knowledge, there is only one method [8] reporting the determination of AL in pharmaceutical formulation: indeed, Demore et al. reported the quantisation of AL in a solution for infusion. Although the degradation of AL in alkaline solution was evaluated, the overall chemical stability of AL was not sufficiently addressed and this method cannot be considered stability indicating. The hydrolytic degradation of AL was studied by Hoffman and Jacobi [9] pointing out its degradation both in acidic and basic conditions: however, no data on the structure of degradation products were reported. Even the role of other stress conditions (e.g. oxidants and light) on chemical stability of AL is at present unknown. An exhaustive study on the stability of AL is demanding as the current International Conference on Harmonisation (ICH) guidelines require that stability analysis should be done by using stability-indicating assay methods, developed and validated after stress testing on the drug under a variety of conditions, including hydrolysis (at various pHs), oxidation, photolysis and thermal degradation [10]. Moreover, the structural characterization and synthesis of the degradation products allow both to establish the degradation pathways and also their quantitative determination in drug substance and product. Hence, in the present work the chemical degradation pathways of AL were established through a forced degradation study and a selective, precise and accurate HPLC-UV method for simultaneous estimation of AL and its degradation products was also developed. The validation of the proposed method was also carried out and its applicability was evaluated in commercial dosage form analysis.

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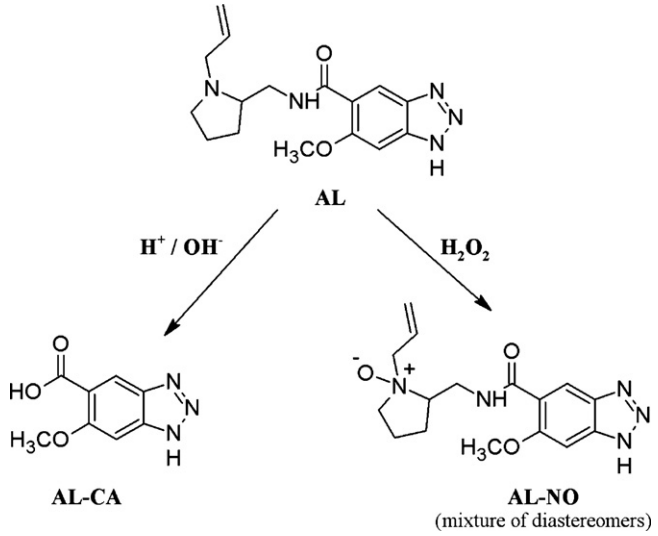

Fig. 1. Degradation scheme of alizapride (AL); alizapride carboxylic acid (AL-CA) and alizapride *N*-oxides (AL-NO).

2. Experimental

2.1. Reagents and chemicals

Methanol (HPLC grade), potassium dihydrogen phosphate, concentrated orthophosphoric acid, iron (III) trichloride hexahydrate, copper sulphate pentahydrate, dichloromethane and 30% (w/w) hydrogen peroxide solution were purchased from Sigma–Aldrich (Milano, Italy). Water (HPLC grade) was obtained from Milli-Q RO system. Limican® ampoules and Limican® tablets, alizapride hydrochloride theoretical dose: 55.8 mg equivalent to 50 mg of AL, and alizapride hydrochloride reference and working standards were obtained as gift samples from Pharmafar S.r.l. (Torino, Italy).

2.2. Instrumentation and chromatographic conditions

2.2.1. HPLC-UV analyses

A Shimadzu HPLC system, consisting in two LC-10AD *Vp* module pumps and a DGU-14-A on-line vacuum degasser, was used. The analyses were carried out on a Polaris C18-A (150 mm × 4.6 mm i.d., 5 μm particle size; Varian, USA) column as a stationary phase. The mobile phase was a mixture of eluant A: aqueous sodium acetate buffer (pH 4.0; 20 mM) and eluant B: CH_3OH using the following gradient: from 0 to 6 min, the composition was increased from 14% to 22% B then maintained for 5 min at a constant flow rate of 1.5 mL min^{-1}. From 11 to 12 min the percentage of eluant B was decreased to 14% and then maintained 10 min for column equilibration.

The eluants A and B were filtered through a 0.2 μm PTFE membrane filter prior the use. A SIL-10AD *Vp* autosampler was used for the injection of samples (20 μL). The SPD-M10A *Vp* photodiode array detector was used to detect AL and the degradation products (AL-CA and AL-NO2) at 225 nm. A ClassVp 5.03 software was used to process the chromatograms. All the analysis were carried out at room temperature.

2.2.2. HPLC–MS/MS analyses

A Thermo Finningan LCQ Deca XP Plus system equipped with a quaternary pump, a Surveyor AS autosampler, a Surveyor PDA detector and a vacuum degasser was used for LC–MS analysis (Thermo Electron Corporation, Waltham, MA, USA).

The chromatographic separation was performed on a Polaris C18-A (150 mm × 4.6 mm i.d., 5 μm particle diameter; Varian, USA) column as a stationary phase maintained at 35 °C.

The mobile phase (flow rate 0.7 mL min^{-1}) was composed of eluant A: ammonium acetate buffer (pH 4; 20 mM) and eluant B: CH_3OH using a linear gradient: from 14% to 20% solvent B in 6 min and then from 20% to 22% B in 10 min, afterward the B fraction was decreased to 14% in 1 min and then maintained for 10 min; the sample injection volume was 20 μL. The eluate was injected into the electrospray ion source (ESI) with a splitting of 40% and the MS and MS/MS spectra were acquired and interpreted using the software Xcalibur® (Thermo Electron Corporation, Waltham, MA, USA). Operating conditions on the ion trap mass spectrometer in positive ion mode were as follows: spray voltage, 5.30 kV; source current, 80 μA; capillary temperature, 350 °C; capillary voltage, 15 V; tube lens offset, 53 V; multipole 1 offset, −8 V; multipole 2 offset, −21.50 V; sheath gas flow (N_2), 60 A.U. Data were acquired in MS/MS product ion scan mode using mass scan range m/z 110–500, and the collision energy was optimized at 33–38%.

2.2.3. Spectroscopic analyses

^1H and ^{13}C experiments were performed on a JEOL Eclipse ECP 300 FT MHz spectrometer (Jeol Ltd., Tokyo, Japan) operating at 7.05 T. Chemical shifts are reported in parts per million (ppm). UV–vis and FT-IR experiments were performed on a Lambda 35 (PerkinElmer, Waltham, MA, USA) and on AVATAR 370 FT-IR (Thermo Fisher Scientific, Madison, WI, USA) spectrophotometers respectively.

2.3. Forced degradation conditions

All degradation studies in solution were done at an AL concentration of 1 mg mL^{-1}. For acidic decomposition study AL was dissolved in 1 M HCl and the solution was left in the dark at 70 °C for 72 h. The degradation in alkaline conditions was done in 0.5 M NaOH and the solution was left in the dark at 70 °C for 48 h. To test the stability in neutral solution AL was dissolved in water and left at 70 °C for 72 h.

For oxidative conditions, the degradation was done in a buffered (pH 6.4) 30% hydrogen peroxide solution and it was left in the dark at room temperature for 48 h. Moreover to evaluate the influence of transition metals, AL solution was treated with 1.5 mM Cu^{2+} or Fe^{3+} at r.t. for 120 h. For thermal degradation, AL was placed in a hot air oven maintained at 60 °C for 48 h. Photodegradation studies were carried out, at room temperature, by exposing a thin layer (50 mg) and an aqueous solution of AL to daylight and UV-light for 72 and 8 h respectively. Before HPLC-UV and HPLC–ESI-MS/MS analyses, acidic and alkaline samples were neutralized and diluted by adding an appropriate volume of mobile phase. For comparison an aqueous solution of AL (1 mg mL^{-1}) was prepared, diluted and analyzed as above.

2.4. Synthesis of putative degradation products

2.4.1. 6-Methoxy-1H-benzotriazole-5-carboxylic acid (AL-CA)

AL (600 mg, 1.7 mmol) was dissolved in 2 M NaOH aqueous solution (40 mL). The solution was left at 70 °C in a steam bath for several hours and monitored by TLC analysis (eluant: $CH_2Cl_2/CH_3OH/CH_3COOH$ 80/20/2). At the end of the reaction (t = 72 h), the mixture was acidified by addition of concentrated HCl and the precipitate was collected by filtration. The crude product was purified by silica column chromatography, using $CH_2Cl_2/CH_3OH/CH_3COOH$ 85/15/1 mixture as eluant to give pure AL-CA (170 mg, 52% yield).

^1H NMR (300 MHz, DMSO-d_6): δ 8.19 (s, aromatic H-7), 7.29 (s, aromatic H-4), 3.88 (s, $-OCH_3$). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ

167.8 (COOH), 157.3 (C-6), 137.7 (C-3a or C-7a), 136.8 (C-3a or C-7a), 122.3 (C-5), 119.9 (CH-4), 93.6 (CH-7), 56.7 (OCH_3). ESI-MS calculated for $C_8H_7N_3O_3$ M = 193.05; Found m/z 192 $[M-H]^-$ ESI-MS/MS m/z 192 $[M-H]^-$ (parent ion), 177, 148. UV–vis (CH_3OH) λ_{max}: 220, 265 (shoulder), 298 nm. FT-IR (KBr) cm^{-1}: 1684.0, 1631.8, 1495.9, 1457.3.

2.4.2. 6-Methoxy-1H-benzotriazole-5-carboxylic acid (1-allyl-1-oxy-pyrrolidin-2-ylmethyl)-amide; diastereomer-2 (AL-NO2) and diastereomer-1 (AL-NO1)

Alizapride hydrochloride (700 mg, 2 mmol) was dissolved in 30% (w/w) H_2O_2 and 0.28 mL (1.4 mmol) of 5 M NaOH solution were added. The mixture was stirred for 24 h at ambient temperature, then neutralized with H_3PO_4 (85%, w/w) and submitted to lyophilisation. The residue was purified by column chromatography using chloroform/methanol/25% ammonium hydroxide (85:15:1.5) as eluant to give pure AL-NO2 (200 mg, 35% yield) and AL-NO1 (30 mg, 4.5% yield).

AL-NO2: ESI-MS calculated for $C_{16}H_{21}N_5O_3$ M = 331.16; Found m/z 332 $[M+H]^+$ ESI-MS/MS m/z 332 $[M+H]^+$ (parent ion), 314, 291, 274, 259, 176. UV–vis (CH_3OH) λ_{max}: 223, 268 (shoulder), 297 nm. FT-IR (KBr) cm^{-1}: 2956.1, 1652.9, 1627.5 1527.5, 1457.3. ^1H NMR (D_2O, 300 MHz, 298 K) and ^{13}C NMR (D_2O, 75.4 MHz, 298 K): see Appendix A.

AL-NO1: ESI-MS calculated for $C_{16}H_{21}N_5O_3$ M = 331.16; Found m/z 332 $[M+H]^+$ ESI-MS/MS m/z 332 $[M+H]^+$ (parent ion), 314, 291, 274, 259, 176. UV–vis (CH_3OH) λ_{max}: 223, 268 (shoulder), 297 nm.

2.4.3. Alizapride hydrochloride and free base

^1H NMR (D_2O, 300 MHz, 298 K) and ^{13}C NMR (D_2O, 75.4 MHz, 298 K): see Appendix A.

2.5. Preparation of standard solutions

Solution A: about 55.8 mg of alizapride hydrochloride reference standard were exactly weighed and dissolved in a 100 mL volumetric flask with a mixture of eluants A and B (86:14, v/v).

Solution B: a combined standard solution of accurately weighed degradation products AL-CA (15 mg) and AL-NO2 (15 mg) was prepared in a 100 mL volumetric flask using a mixture of eluants A and B (86:14, v/v) as solvent. The final concentration of AL-CA and AL-NO2 was 150 $\mu g\,mL^{-1}$.

2.5.1. Preparation of analytical samples

Limican® ampoules (AL assay): the contents of 20 ampoules were mixed and 2.0 ml of the solution (sample solution) were diluted to 100 mL in a volumetric flask, with a mixture of eluants A and B (86:14, v/v). The resulting solution was further diluted 1:10 using the same solvent to get a concentration of 50 $\mu g\,mL^{-1}$ of AL (theoretical value). After filtration through 0.2 μm PTFE membrane filter, the sample was analyzed by LC–UV.

Limican® ampoules (degradation products assay): 3.0 mL of the sample solution were diluted to 50 mL in a volumetric flask, with a mixture of eluants A and B (86:14, v/v) and treated as reported above.

Limican® tablet (AL assay): 20 tablets were finely pulverized in a mortar; 240 mg of the powder were exactly weighed and transferred into a 100 mL volumetric flask; it was made up to the mark with a mixture of eluants A and B (86:14, v/v). The mixture was sonicated and stirred for 5 and 10 min respectively. Finally 5.0 mL of the mixture were diluted 1:10 using the same solvent. After filtration through 0.2 μm PTFE membrane filter, the sample was analyzed by HPLC.

Limican® tablet (degradation products assay): 360 mg of finely pulverized tablets were exactly weighed and transferred to a 50 mL volumetric flask which is filled up with a mixture of eluants A and B (86:14, v/v).

2.6. Validation procedure

2.6.1. System suitability

The system suitability parameters resolution (Rs), area repeatability and asymmetry factor (As) were calculated as previously reported [11].

2.6.2. Selectivity

To assess the method selectivity reconstructed tablets and ampoules formulations without AL were prepared with the excipients used for Limican® commercial preparations. For HPLC analysis the solution was prepared using the same procedure of analytical sample. Moreover to evaluate the influence of AL-CA and AL-NO1/AL-NO2, a standard solution was prepared as reported above except the addition of the degradation products. After HPLC analyses, resolution factors were calculated as previously reported [11].

2.6.3. Robustness

To determine the robustness two parameters were varied: pH and percent composition of eluants. The influence of the pH of the mobile phase was studied by analyzing the standard mixture of AL, AL-CA and AL-NO2 at six different values: 2.5, 3.0, 3.2, 3.5, 3.8 and 4.0. The effect of the pH change was evaluated by calculating the resolution factors. The influence of mobile phase composition was determined by varying the initial (13–14–15%) and final (20–22–24%) percentages of eluant B.

2.6.4. Linearity

Linearity of the method was evaluated at five equispaced concentration levels by diluting the standard solutions to give solutions over the ranges 50–150% and 0.05–1.2% of the target concentration for alizapride and degradation products respectively. These were injected in triplicate and the peak areas were inputted into a Microsoft Excel® spreadsheet program to plot calibration curves. To satisfy basic requirements such as homoscedasticity and linearity, the Bartlett test and the lack-of-fit-test were respectively performed at the 95% significance level.

2.6.5. Precision

Precision was evaluated in terms of intra-day repeatability and inter-day reproducibility.

The intra-day repeatability was investigated using six separate sample solutions prepared, as reported above, from the freshly reconstructed tablet and ampoules formulations at 100% of the target level. Each solution was injected in triplicate and the peak areas obtained were used to calculate means and RSD% values.

The inter-day reproducibility was checked on four different days, by preparing and analysing in triplicate four separate sample solutions from the reconstructed formulations at the same concentration level of intra-day repeatability; the means and RSD% values were calculated from peak areas.

2.6.6. Accuracy

To assess accuracy, freshly prepared placebo of the AL pharmaceutical formulations was spiked with various amounts of AL at 80, 100 and 120% of the target concentration and AL-CA and AL-NO2 at 0.05, 0.2, 0.5% of the target concentration. Each solution was injected in triplicate and the peak areas were used to calculate means and RSD% values and compared with those obtained with standard solutions.

3. Results and discussion

3.1. Synthesis of AL degradation products

Literature data and the presence of a tertiary amine function in the structure of AL suggested that from hydrolytic and oxidative degradation pathways the putative degradation products alizapride carboxylic acid and diastereomeric alizapride *N*-oxides could be formed. The synthesis of AL-CA was easily accomplished from hydrolysis of AL in alkaline medium. Negative ESI-MS data showed the presence of a pseudomolecular ion at 192 *m*/*z* indicating the loss of the 1-allylpyrrolidin-2-ylmethylamine moiety through amide cleavage. The full characterization of its structure was achieved by $^{1}H/^{13}C$ NMR and FT-IR analyses. As expected, in the ^{1}H NMR spectrum the signals of H-7 and H-4 aromatic protons at 8.19, 7.29 δ and methoxy function at 3.88 δ were found. Moreover in the ^{13}C NMR spectrum the signal at 167.7 δ confirmed the presence in the structure of a carboxylic function which has been also evidenced by the carbonyl absorbing band at 1684 cm^{-1} in IR spectrum. The synthesis of alizapride *N*-oxides (AL-NO) was performed using hydrogen peroxide as oxidizing reagent. Since the reaction was conducted starting from alizapride hydrochloride an equivalent of sodium hydroxide was added to promote the oxidation. Two products were formed (AL-NO1 and AL-NO2; ~10/90 ratio based on HPLC-UV chromatogram), the less polar being the major one. Both AL-NO1 and AL-NO2 positive ESI-MS spectra showed the presence of a pseudomolecular ion at *m*/*z* 332, indicating the insertion of an oxygen atom in the alizapride structure, and identical fragment ion patterns. These features suggested that AL-NO1 and Al-NO2 were diastereomers. Since AL-NO2 was the most abundant isomer, it can be considered an indicator of AL oxidative stability; hence its $^{1}H/^{13}C$ NMR spectroscopic characterization was performed assigning the corresponding resonances with the help of double resonance and 2D (COSY and HMQC) experiments.

In order to help with the structural determination of AL-NO2, the spectra of alizapride and its hydrochloride were registered too; amazingly, no NMR data are available in the literature for alizapride and its hydrochloride salt. The comparison of the NMR spectra of these three compounds was extremely useful for the localization of the oxidation site. Significant differences between alizapride and AL-NO2 were detectable in the ^{13}C NMR spectra. Downfield shifts, observed for C-2′ (+7 ppm), C-5′ (+12.9 ppm), C-7′ (+11.5 ppm), clearly indicated that the structural modification was localized to N-1′, the latter being the only atom sharing connection with these carbon atoms. ^{1}H NMR spectra were less useful, even if some minor chemical shift differences were found for H-2′, H-3′, H-5′, H-7′ and H-8′, all corroborating the oxygen atom position on N-1′. It is worth noting that the oxidation-induced chemical shift variations closely match those observed on protonation (i.e. alizapride hydrochloride): this is not surprising as the nitrogen atom undergoes a similar stereoelectronic change in both transformations.

3.2. Forced degradation study and structural characterization of degradation products

The degradation of AL in both alkaline and acidic conditions was previously reported [9]; however, no structure characterization of the related degradation products was obtained and the influence of other stress conditions (e.g. oxidative and photolytic) on the chemical stability of AL was not studied. In the forced degradation study all the stress conditions required by ICH guidelines were included; moreover in order to avoid unrealistic degradation pathways, the conditions were adjusted to obtain a 10–25% degradation of the parent compound. As a matter of fact, in 1 M HCl and 0.5 M NaOH the degradation of AL took place with the formation of the more polar degradation product AL-CA as reported in HPLC-UV chromatograms (Figs. 2A and 3A). HPLC–ESI-MS/MS analyses (ion at *m*/*z* 194) revealed the presence of the same degradation product both in alkaline and acidic sample solutions (Figs. 2B and 3B), even if in the acidic samples the peak of AL-CA appears very broad. The structure of 6-methoxy-*1H*-benzotriazole-5-carboxylic acid was assigned to the degradation product based on ESI-MS data and comparison with a synthetic reference sample of AL-CA. The oxidative degradation pathway was studied using two different protocols: in the presence of hydrogen peroxide, as reported in HPLC-UV chromatogram (Fig. 4A), two degradation products were formed (AL-NO1 and AL-NO2) being AL-NO2 the major one. Similarly, HPLC–ESI-MS/MS analysis (*m*/*z* 332) (Fig. 4B), showed two peaks with the same retention time of those observed in the HPLC-UV chromatogram. Moreover the pseudomolecular ion at *m*/*z* 332 indicated the insertion of an oxygen atom in the structure of AL suggesting that AL-NO1 and AL-NO2 were diastereomers. Taking into account the lack of reactivity towards oxidizing agents of the benzotriazole scaffold, and data arising from ESI-MS/MS experiments the formation of *N*-oxide of pyrrolidine moiety was assumed. Finally an authentic sample of AL-NO2 showed the

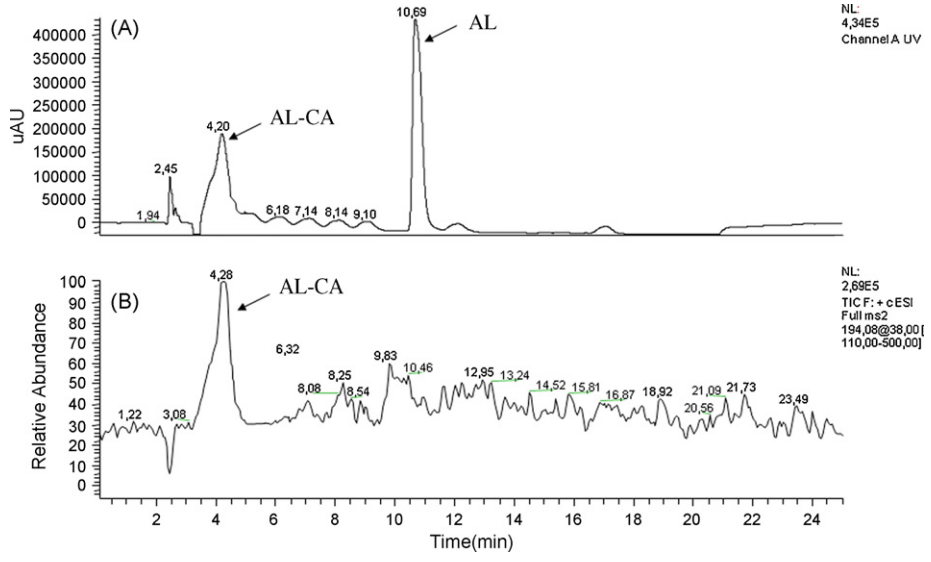

Fig. 2. HPLC-UV–ESI-MS/MS chromatograms of alizapride degradation in 1 M HCl (*t* = 72 h): (A) UV trace and (B) positive MS/MS trace (*m*/*z* 194).

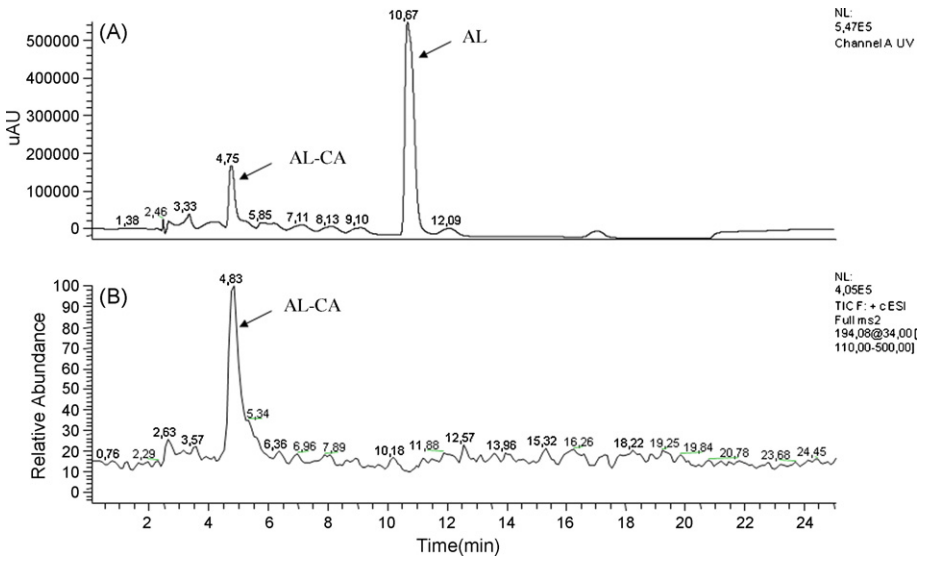

Fig. 3. HPLC-UV–ESI-MS/MS chromatograms of alizapride degradation in 0.5 M NaOH (t = 48 h): (A) UV trace and (B) positive MS/MS trace (m/z 194).

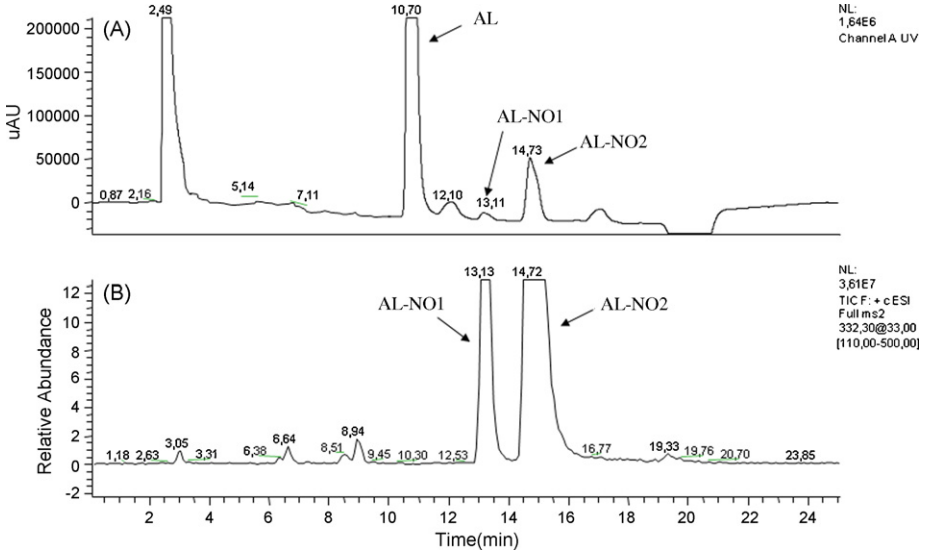

Fig. 4. HPLC-UV–ESI-MS/MS chromatograms of alizapride degradation in 30% H_2O_2; (A) UV trace and (B) positive MS/MS trace (m/z 332).

same chromatographic and mass spectral data of the degradation product. These considerations allowed assigning the structure of 6-methoxy-*1H*-benzotriazole-5-carboxylic acid (1-allyl-1-oxy-pyrrolidin-2-ylmethyl)-amide to AL-NO2. AL was found to be stable in other oxidative stress conditions: indeed in Fe^{3+} and Cu^{2+} aqueous solutions AL was not oxidized. Finally, light and thermal stress conditions did not promote the formation of degradation products.

3.3. Method development

The data obtained from forced degradation study allowed to develop a single HPLC assay for the analysis of AL, in the presence of its degradation products AL-CA and AL-NO2. As a matter of fact the analytes showed different structural features: a tertiary amine, a carboxylic group and a polarized *N*-oxide function were present in AL, AL-CA and AL-NO2 respectively. Several types of stationary phases (C-18, C-8 and phenyl-reverse phases) with various mobile phases containing acetonitrile or methanol–aqueous phosphate buffers were employed during the method development. In almost every studied system, the separation of all analytes was not obtained (data not shown). To maximize polar retention and selectivity, a polar embedded stationary phase was used allowing a better separation of the analytes in an acceptable runtime. In particular, we have chosen a Polaris C18-A phase in which there is a hydrophilic linkage between silica particles and hydrophobic alkyl chains. These features conferred the desired selectivity to the method. Moreover, an acidic phosphate buffer was firstly employed to minimize the interaction between tertiary amine function and stationary phase silanol functions. However, the evaluation of the method robustness showed a dramatic influence of the pH on the resolution of AL-CA and AL analytes, as reported in Table 1. Even if the resolution values within the pH range 3.0–3.5 were all acceptable, a pH 4 acetate buffer was preferred as eluant, because of the smaller resolution variability observed

Table 1
Resolution of adjacent peaks AL-CA/AL *vs* eluant pH.

Eluant pH	2.5	3.0	3.2	3.5	3.8	4.0
Rs (AL-CA/AL)	Overlap	2.8	3.9	8.3	7.0	7.7

Table 2
System suitability parameters.

	AL	AL-CA	AL-NO2	CDER acceptance criteria
Asymmetry	1.0	1.0	1.0	≤2.0
Resolution	AL/AL-CA > 5		AL/AL-NO2 > 5	>2
Repeatability of peak area	3,767,235 (0.4)	286,738 (2.1)	173,023 (2.1)	≤1.0%[a]

The concentrations of AL, AL-CA and Al-NO2 were 50, 3.0 and 3.0 μg mL^{-1} respectively. The figures in brackets represent %RSD values for six replicates.
[a] This acceptance criteria only applied to AL.

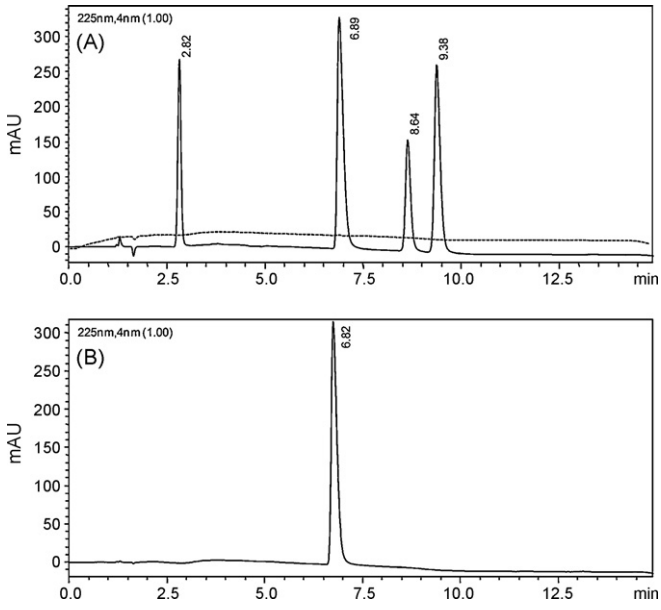

Fig. 5. (A) HPLC-UV chromatogram of a combined standard solution containing AL, AL-CA, and alizapride *N*-oxides (AL-NO1 and AL-NO2). UV detection at 225 nm. AL (Rt = 6.89 min), AL-CA (Rt = 2.82 min), AL-NO1 (Rt = 8.64 min), and AL-NO2 (Rt = 9.38 min). The dotted line represents the chromatogram of the placebo solution. (B) HPLC chromatogram of a stability study sample (tablets; *t* = 24 months). AL; Rt = 6.82 min.

in the pH range 3.5–4.5. On the contrary, no difference in method performances was observed applying smaller variations to the initial and final mobile phase composition. Accordingly, the starting mobile phase was constituted by eluant A: aqueous sodium acetate buffer (20 mM, pH 4.0) and eluant B: methanol, the A:B ratio being 86:14, and the flow rate 1.5 mL min^{-1}. The elution was performed in gradient mode as reported in the experimental. All these features provided chromatograms with good peak shape required for the simultaneous analysis of a AL, AL-CA, AL-NO2 mixture with an acceptable time of analysis (Fig. 5A). For quantitative determination, detection of AL, AL-CA and AL-NO2 was performed at 225 nm.

3.4. Method validation

The developed method was validated [12], as described below, for the following parameters: system suitability, specificity, linearity, precision, accuracy and LOD/LOQ.

3.4.1. System suitability

As system suitability test is an integral part of chromatographic methods development and it is used to verify that the system is adequate for the analysis to be performed, the parameters for AL, AL-CA and AL-NO2 were evaluated. The suitability of the chromatographic system was demonstrated by comparing the obtained parameter values, reported in Table 2, with the acceptance criteria of the CDER guidance document [13].

3.4.2. Selectivity

Selectivity is the ability of an analytical method to assess unequivocally the analytes in the presence of components that are present in the sample matrix. The representative chromatogram (Fig. 5A) of the placebo solution constituted by excipient blend showed no peak interfering with analytes; moreover the adjacent chromatographic peaks AL-CA/AL and AL/AL-NO2 were separated with resolution factors >5. It is worth of mention that, AL-NO1 was separated from other analytes (Rs = 5.7 AL/AL-NO1; Rs = 2.8 AL/NO1/AL-NO2) but it was not further considered in the validation process. Overall, these data demonstrated that the excipients and the degradation products did not interfere with the alizapride peak, indicating selectivity of the method.

3.4.3. Linearity

The linearity of an analytical procedure is its ability, within a given range, to obtain test results which are directly, or through a mathematical transformation, proportional to the concentration of analyte. Five concentration levels within 50–150% of the target concentration range for AL were considered to study the linearity. For degradation products AL-CA and AL-NO2 a range value of 0.05–1.20% was used. Since the Bartlett test evidenced no significant difference ($p > 0.05$) among the variance values of replicates at different concentration levels the best fit was obtained using an unweighed linear regression model. The linearity was observed in the expected concentration range, demonstrating its suitabil-

Table 3
Five levels calibration graph of alizapride: unweighted linear regression $y = ax + b$; three replicates for each level ($n = 15$).

Concentration[a] (μg mL^{-1})	Range (μg mL^{-1})	*a* (SE[b])	*b*	*b* C.I.[c]	*F*	r^2	%RSD[d]
AL	25–75	73019.9 (381.2)	39073.7	−4597.7 to 82745.1	36697.5 $p < 0.01$	0.9996	0.7
AL-CA	1–15	97152 (423.4)	−7988.7	−14997.7 to −979.7	52636.2 $p < 0.01$	0.9997	2.9
AL-NO2	1–15	55791.8 (257.1)	−3534.4	−7789.7 to 720.9	47094.3 $p < 0.01$	0.9997	3.1

[a] Target concentration corresponding to 100%.
[b] Standard error.
[c] 95% confidence interval.
[d] % relative residual standard deviation.

Table 4
Intra-day and inter-day precision data for alizapride and its degradation products.

Analyte	% of target concentration	Intra-day variation (%RSD)	Inter-day variation (%RSD)
AL	100[a]	0.8 (n = 12)	1.0 (n = 12)
AL-CA	0.2[b]	1.3 (n = 12)	1.7 (n = 12)
AL-NO2	0.2[b]	2.1 (n = 12)	4.8 (n = 12)

[a] 100% of target concentration was equivalent to 50 μg mL^{-1} of AL.
[b] 0.2% of target concentration was equivalent to 3 μg mL^{-1} of AL-CA and AL-NO2.

Table 5
Accuracy: recovery data for alizapride and its degradation products.

% of targeting concentration[a]	AL % recovery	% of targeting concentration[b]	AL-CA % recovery	AL-NO2% recovery
80	100.9 (0.4)	0.05	99.6 (0.5)	97.5 (1.2)
100	100.5 (0.3)	0.2	98.6 (0.3)	96.8 (2.5)
120	99.4 (0.6)	0.5	100.9 (2.1)	96.2 (3.4)
Average recovery	100.3 (0.4)	Average recovery	99.7 (1.0)	96.8 (2.4)

[a] 100% of target concentration is equivalent to 50 μg mL^{-1} of AL.
[b] 0.2% of target concentration is equivalent to 3 μg mL^{-1} of AL-CA and AL-NO2.
The figures in brackets represent RSD% values for three replicates.

ity for analysis. The results of the regression statistics obtained for AL, AL-CA and AL-NO2 were reported in Table 3. The square of the correlation coefficient ($r^2 > 0.999$) demonstrated a significant correlation between the concentration of analytes and detector response; however, it was neither a proof of linearity, nor a useful measure of the calibration variability. Hence the lack-of-fit-test was performed on these data; the significance values ($p > 0.05$) obtained for all analytes indicated that a linear regression model provides a good interpolation of the experimental data. Moreover, the evaluation of residual plot confirmed that underlying assumption like homoscedasticity was met as well as the goodness of fit of the regression model. Finally the confidence interval of the y-intercepts includes zero and the relative residual standard deviations, expressed as percentage, were calculated and used to assess the precision of the regression; the values were 0.7, 2.9 and 3.1% for AL, AL-CA and AL-NO2 respectively.

3.4.4. Precision

The precision of an analytical procedure expresses the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The repeatability (intra-day precision) refers to the use of analytical procedure within a laboratory over a short period of time using the same operator with the same equipment. Intermediate precision (inter-day precision) involves estimation of variations in analysis when a method is used within a laboratory on different days, by different analysts. The results obtained are shown in Table 4. In all instances the %RSD values were less than 2%.

3.4.5. Accuracy

The accuracy of an analytical method is the closeness of the test results to the true value. It has been determined by application of the analytical procedure to recovery studies, where known amount of standard is spiked into the placebo. The results of accuracy studies from standard solution and excipient matrix were shown in Table 5; recovery values demonstrated that the method was accurate within the desired range.

3.4.6. LOD–LOQ

The LOD of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified. It is expressed as a concentration at a specified signal-to-noise ratio, usually 3. The limit of detection was determined, by injecting progressively low concentration, only for the degradation products AL-CA and AL-NO2: the concentration for both AL-CA and AL-NO2 was 0.3 μg mL^{-1}. The related LOQ values were then calculated obtaining 1 μg mL^{-1} both for AL-CA and AL-NO2.

3.5. Analysis of commercial tablets and ampoules formulations

HPLC chromatogram obtained from a tablets sample employed in long-term stability study (t = 24 months) was reported in Fig. 5B. The HPLC trace allowed excluding the formation of the degradation products AL-CA and AL-NO2. Identical results were obtained by analysis of alizapride ampoules formulation.

4. Conclusions

In the present work the chemical stability of alizapride was investigated and a degradation scheme was proposed on the basis of a forced degradation study. The identification and synthesis of the two main degradation products allowed the development and validation of a stability-indicating HPLC-UV method for the determination of AL and degradation products in the active ingredient and its pharmaceutical formulations. The complete separation of the analytes was accomplished in 10 min and the method has been successfully used to perform long-term and accelerate stability studies of AL formulations.

Acknowledgement

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Appendix A. Appendix A

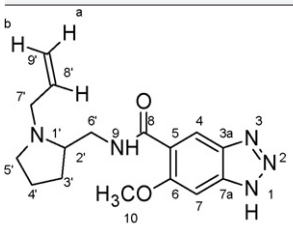
See Table A1.

Table A1
^{1}H NMR and ^{13}C NMR chemical shifts (ppm) of AL, AL-HCl and AL-NO2.

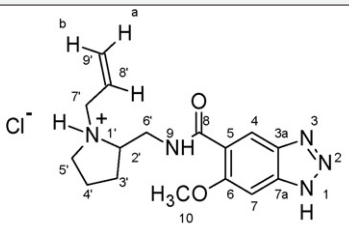
Position	AL		AL-HCl		AL-NO2	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	–	–	–	–	–	–
2	–	–	–	–	–	–
3	–	–	–	–	–	–
3a	–	141.8/137.5	–	136.2/135.8	–	136.4/135.8
4	7.69 (s)	119.5	7.88 (s)	119.8	7.67 (s)	119.5
5	–	118.4	–	120.1	–	120.7
6	–	154.6	–	156.3	–	156.3
7	6.48 (s)	93.7	6.78 (s)	92.0	6.49 (s)	92.0

Table A1 (*Continued*)

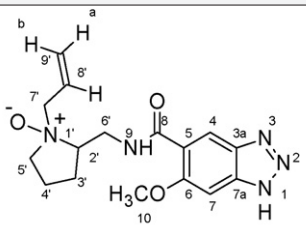
Position	AL		AL-HCl		AL-NO2	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
7a	–	141.8/137.5	–	136.2/135.8	–	136.4/135.8
8	–	168.1	–	167.6	–	166.8
9	–	–	–	–	–	–
10	3.43 (s)	55.7	3.76 (s)	56.2	3.58 (s)	56.0
1′	–	–	–	–	–	–
2′	3.41–3.27 (m)	66.0	3.73–3.61 (m)	66.8	3.72 (m)	73.0
3′	2.02 (m) 1.66 (m)	27.4	2.32 (m) 1.93 (m)	27.4	2.15 (m) 1.93–1.83 (m)	25.6
4′	1.92–1.76 (m)	21.9	2.18–2.00 (m)	21.9	2.02 (m) 1.93–1.83 (m)	19.1
5′	3.41–3.27 (m) 2.86 (dt; 11.4, 7.8)	53.8	3.73–3.61 (m) 3.25 (dt; 10.9, 6.9)	54.2	3.51–3.41 (m) 3.35 (m)	66.7
6′	3.41–3.27 (m)	40.1	3.73–3.61 (m)	40.1	3.86–3.78 (m) 3.51–3.41 (m)	37.9
7′	3.66 (dd; 13.2, 6.7) 3.41–3.27 (m)	56.6	4.00 (dd; 12.9, 6.9) 3.78 (m)	57.0	4.05 (dd; 13.0, 6.6) 3.86–3.78 (m)	68.1
8′	5.73 (ddt; 17.0, 10.0, 7.0)	127.7	5.93 (m)	126.7	6.00 (ddt; 17.3, 10.4, 7.1)	127.2
9′	5.73–5.27 (m)	124.8	a 5.50 (d; 10.2) b 5.56 (d, 17.0)	125.9	a 5.46 (d; 10.6) b 5.45 (d; 17.1)	125.8



AL



AL• HCl



AL-NO2

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