Development of the Analytical Method for LC-MS Detection of Unknown Degradation Product of Alprazolam

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The stability of alprazolam as a pure substance and in the tablets was studied. The gradient HPLC chromatographic conditions were described for LC-MS identification of an impurity. Employing this chromatographic method, the stability of alprazolam under conditions of dry and wet heat was observed. The unknown degradation product was identified using MS detection.

Alprazolam is a member of a new class of 1,4-benzodiazepines characterized by high potency, oral activity, and low toxicity. Alprazolam is a compound acting as a partial agonist at benzodiazepine receptors. It has shown neuropsychopharmacological effects. Classic benzodiazepines are known to produce anxiolytic, anticonvulsive, sedative, myorelaxant effects, to potentiate alcohol and to impair cognitive processes at overlapping dose ranges, while alprazolam appears to be predominantly anxiolytic. It is also used as a sedative-hypnotic agent. Alprazolam is a good hypnotic drug for management of insomnia, but overdose could be fatal [1—3].

Benzodiazepines are extensively prescribed for elderly individuals. The short-acting benzodiazepine alprazolam was one of the most widely used benzodiazepines in the last year. Alprazolam is important to toxicologists because of its frequent use and its potential to affect a person's ability to drive a motor vehicle or to fly an airplane [4, 5].

Drug stability constitutes an important current subject of investigation because the degradation process can result in a loss of the potency of the drug and also in adverse effects due to the formation of minor toxic degradation products. Hydrolysis of the benzodiazepinone ring is one of the most frequently observed degradation routes for benzodiazepines. A reversible benzodiazepine ring-opening under aqueous acidic conditions has been described for alprazolam earlier [6].

Alprazolam undergoes a facile 1,4-benzodiazepine ring-opening reaction in an acidic aqueous solution to form a benzophenone compound. Its reverse cyclization reaction to alprazolam occurs when acidic solution is neutralized [7]. Other papers describe ring-opening of benzodiazepines and thermal stability of alprazolam [8, 9].

The aim of this paper was to elaborate the optimum fast and reliable method of LC in conjunction with MS that would enable to detect the part of degradation products of alprazolam.

EXPERIMENTAL

Chemicals used (all anal. grade) were: Alprazolam (Léčiva a.s.), Neurol 0.25 tbl. (Léčiva a.s.) as the substrates, acetic acid, formic acid (Lachema, CR), methanol HPLC, ammonium formate, ammonium acetate, acetonitrile HPLC (Sigma-Aldrich, Germany), water MilliQ grade.

HPLC analysis proceeded at these chromatographic conditions: Column Luna Phenyl-Hexyl with parameters 250 mm × 4.6 mm; 5 μm (Phenomenex). Mobile phase used: *1.* ammonium acetate buffer (7.7 g + 900 cm^3 H_2O) or ammonium formate buffer (0.13 g + 200 cm^3 H_2O) with modified pH 3.2—6.2 by formic acid and acetonitrile; *2.* ammonium acetate buffer (7.7 g + 900 cm^3 H_2O) or ammonium formate buffer (0.13 g + 200 cm^3 H_2O) with modified pH 3.2—6.2 by formic acid and methanol in different ratio and by different gradient. Column temperature: 40 °C, flow rate: 2.0 cm^3 min^{-1} or 0.5 cm^3 min^{-1}, detection at 254 nm. HPLC system – pump, UV detector, thermostat from Hewlett—Packard 1050 with HP ChemStation software.

LC/MS/MS system – Perkin—Elmer series 200

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Micro Pump, autosampler, API 3000 Applied Biosystem, an UV VIS detector – 785A and Triple Quadrupole LC/MS/MS Mass Spectrometer with electrospray ionization. The separation was performed on the same type of column mentioned above. The mobile phase was composed of ammonium formate (0.01 M, pH 4.2) and methanol. A linear gradient rate was programmed as shown in Table 2. The mass determination was made in positive mode in the mass range of 70.0—1200.7. The data from this system were analyzed using Mass Chrom 1.1 Software.

Samples

Dry heating. Eight tablets crushed into fine powder or alprazolam (substance) – 10.06 mg were heated at 90 °C for 24 h and then the volume was filled up to 10.0 cm^3 by 70 % MeOH. The volumetric flask was kept in an ultrasonic bath for 15 min and then it was shaken for 10 min. After this process the sample was centrifuged for 10 min at 5000 min^{-1}. 100 mm^3 from this sample were applied to column.

Wet heating. Eight tablets crushed to fine powder or 10.10 mg of alprazolam substance were wetted by 100 mm^3 of distilled water, then they were heated at 90 °C for 24 h. Further process was the same as at dry heating.

In the case of substance heating too small area of peak with retention time about 8 min was found on chromatographic record.

All samples were analyzed at chromatographic conditions as shown in Table 1.

It was necessary to replace the acetate buffer with the formate buffer for LC-MS evaluation and it was necessary to decrease the flow rate (0.5 cm^3 min^{-1}) and these chromatographic conditions are shown in Table 2.

Table 1. Chromatographic Conditions for the Analysis of the Samples (Dry, Wet Heating)

Time/min	w(Mobile phase A)/%	w(Mobile phase B)/%
0—15	80	20
15—35	80→10	20→90
35—40	10	90
40—45	10→80	90→20
45—50	80	20

Mobile phase A: 200 cm^3 of ammonium acetate, pH 3.2 + 60 cm^3 of methanol.
Mobile phase B: Methanol.
Flow rate: 2.0 cm^3 min^{-1}.

Table 2. Chromatographic Conditions for the Identification of Impurity

Time/min	w(Mobile phase A)/%	w(Mobile phase B)/%
0—20	99→80	1→20
20—30	80	20
30—35	80→10	20→90
35—50	10→1	90→99
50—55	1	99
55—60	1→99	99→1
60—65	99	1

Mobile phase A: ammonium formate (0.01 mol dm^{-3}), pH 4.2/methanol (φ_r = 44:56).
Mobile phase B: Methanol.
Flow rate: 0.5 cm^3 min^{-1}.

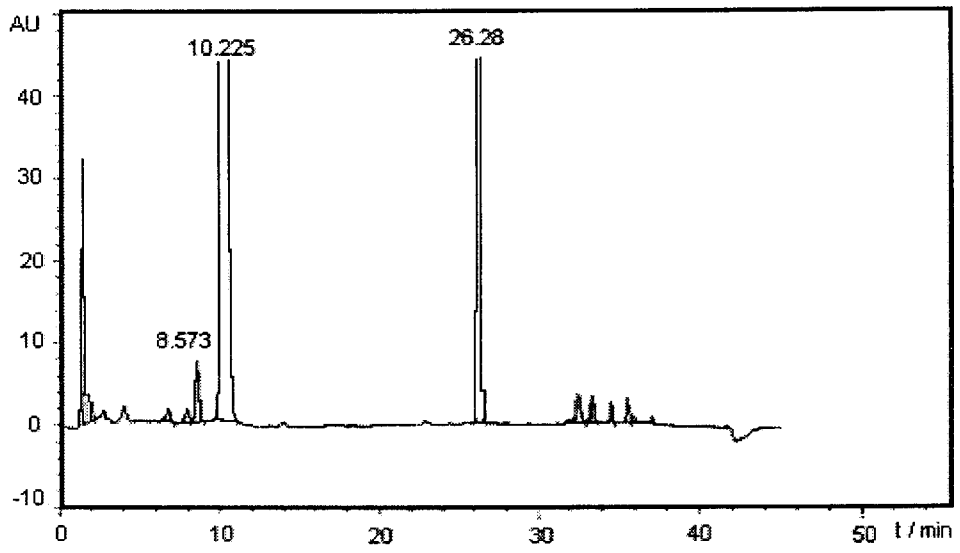


Fig. 1. The peak with a retention time of 10.23 min is alprazolam, the peak with a retention time of 26.28 min is 7-chloro-5-phenyl-1-methyl[1,2,4]triazolo[4,3-*a*]quinoline-4-amine and the peak with a retention time of 8.57 min is impurity.

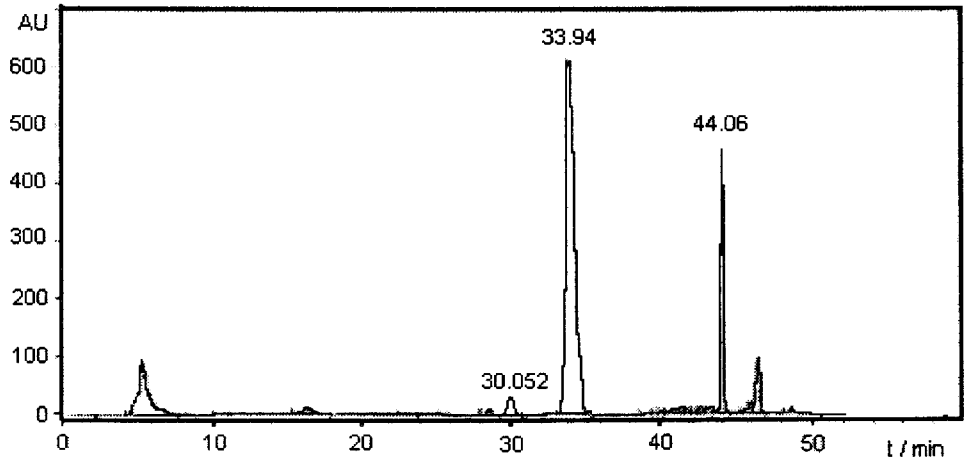


Fig. 2. The peak with a retention time of 30.05 min is impurity, the peak with a retention time of 33.94 min is alprazolam and the peak with a retention time of 44.06 min is 7-chloro-5-phenyl-1-methyl[1,2,4]triazolo[4,3-*a*]quinoline-4-amine.

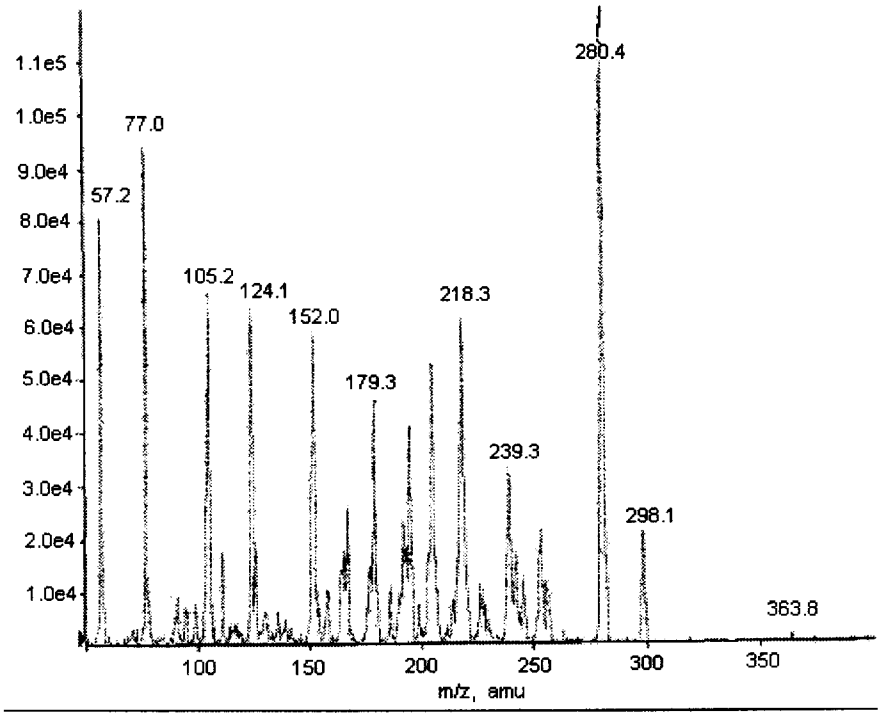


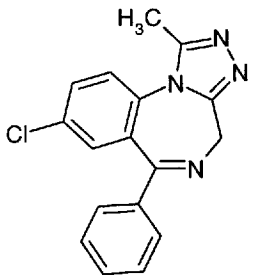
Fig. 3. MS record of impurity of alprazolam.

RESULTS AND DISCUSSION

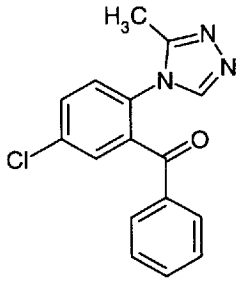
Several mobile phases were tested. When the lower value of pH of the buffer (pH 3.2) was used, the peak of alprazolam was melted into the peak of the impurity. When methanol was replaced with acetonitrile, it was not possible to identify which peak is the impurity, because several peaks with similar area were observed on the chromatographic record. The chromatographic conditions presented in Table 1 were suitable for the selection of samples with the greatest volume of impurity (Fig. 1) and then the chromatographic conditions were modified for LC-MS detection. At the end the method presented in Table 2 was chosen because the peak of impurity was separated enough from the peak of alprazolam as shown in Fig. 2.

The heating effect on the substance or the tablets was observed in chromatogram as a fraction of the peak area. Only 0.27 % of the impurity has been found after the dissolution of 8 tablets without heating in this sample. It was necessary to increase this fraction so as this peak could be identified at the modified chromatographic conditions. When the sample (8 tablets) was heated at 90 °C for 24 h, there was obtained 0.92 % of impurity. By wet heating there was found 1.59 % of the peak area of impurity. The effect

of substance heating was inconsiderable. The sample with 8 tablets after effect of wet heating was used for modification of the HPLC method for LC-MS detection. Following MS/MS fragmentation of M + 1 ion ($m/z = 298$) demonstrated the presence of characteristic peaks with $m/z = 105$ and $m/z = 77$ as shown in Fig. 3. The impurity was identified as a degradation product of alprazolam with the structure shown in structural formulae.



Alprazolam



Degradation product

CONCLUSION

Degradation product of alprazolam with molar mass 297 g mol^{-1} with one Cl atom and odd number of N atoms was identified. Its structure is shown in structural formulae.

This degradation product was detected in the most quantity after the wet heating of tablets. This paper brings the knowledge that the impurity is the degradation product of alprazolam.

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