A New Bioanalytical Method for the Determination of Alprazolam in Human Plasma

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A new liquid chromatography-tandem mass spectrometry method has been developed and validated for the determination of alprazolam in human plasma, using lorazepam as internal standard. Detection was performed using positive ion electrospray tandem mass spectrometry on an Agilent 1200 Triple Ouad 6410A system. The mass transition ion-pair was followed as m/z 309→ 281 for alprazolam and m/z 321→ 275 for lorazepam, in multiple-reaction monitoring mode. The plasma samples were extracted with tert-buthyl methyl ether and separated by liquid chromatography. Alprazolam and the internal standard were separated on a Zorbax SB-CN column using a mobile phase consisting in 70:30 (v/v) mixture of acetonitrile and 0.5% formic acid aqueous solution, with a flow rate of 0.8 mL/min. The proposed method has been validated for the concentration range in between 0.1 and 50 ng/mL and a correlation coefficient of 0.9991. The precision and accuracy were within 12% for intra-day and inter-days assays. The overall recoveries for alprazolam and lorazepam were 76% and 88%, respectively. The overall time of one analysis was 4 minutes. The assay has proven to be sensitive, specific and reproducible, suitable for the quantitative determination of alprazolam in human plasma.

Keywords: alprazolam, liquid chromatography, mass spectrometry, plasma.

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazole [4,3,-α]-[1,4] benzodiazepine) (fig. 1) is a new generation 1.4-benzodiazepine belonging to the nitrogen heterocycle compounds class. It is a benzodiazepine mainly used to treat anxiety disorders. On a short time basis it is used to palliate symptoms of anxiety or anxiety associated to symptoms of depression. Besides this, alprazolam is also used to treat panic disturbances with or without agoraphobia [1–5].

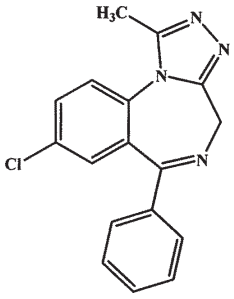

Fig.1. Chemical structure of alprazolam

Several methods for the quantitative determination of alprazolam in human plasma have been reported. Mainly, liquid chromatography methods with UV detection were described [6-10].

LC-MS methods have been widely accepted as the most used method for the quantitative determination of drugs due to their sensitivity and specificity [13-16].

The present study reports a sensitive, specific and reproducible liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of alprazolam in human plasma (EDTA-K_3, anticoagulant), using lorazepam as internal standard. This assay provides improved extraction recovery (76.632-81.395%), a wide linear dynamic range (0.1-50 ng/mL) and a lower limit of quantification of 0.1 ng/mL. The developed assay was successfully applied to a pharmacokinetic study after oral administration of alprazolam to healthy volunteers.

Experimental part

Chemicals and reagents

The standard Alprazolam (batch number 2) used in this study was supplied by Official European Pharmacopeia, with a purity of 100.00 %. The internal standard Lorazepam (batch number 1b) was supplied by Official European Pharmacopeia with a purity of 100.00 %. All solvents and other chemicals were HPLC grade provided by Merck, Germany. The human plasma was obtained from Center for Blood Drawing and Preservation Iaşi, Romania.

Preparation of stock solutions, calibration standards and quality control samples

The reference substance, alprazolam, accurately weighed, was dissolved in methanol, obtaining 1 mg/mL stock solution. Alprazolam intermediate solutions (50 μg/mL, 5 μg/mL and 0.1 μg/mL) were prepared by diluting the alprazolam stock solution with methanol. The alprazolam intermediate solutions were diluted with methanol to give the alprazolam working solutions for calibration standards and quality control samples, at the following concentrations: 10, 20, 250, 500, 1000, 2000, 3000, 4000, 5000 ng/mL (the working solutions for calibration standards) and 30,1250,3500,10, 5000 ng/mL (the working solutions for quality control samples).

Lorazepam stock solution (0.2 mg/mL) was prepared by accurately weighing the lorazepam reference substance and dissolution in methanol to give a final concentration of 0.2 mg/mL. A lorazepam intermediate solution (1000 ng/mL) was prepared by diluting the lorazepam stock solution with methanol. A lorazepam working solution (100 ng/mL) was prepared by diluting 1 mL lorazepam intermediate solution (1000 ng/mL) up to 10 mL with distilled water.

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Working solution for calibration standard			Plasma blank	Calibration standard
Theoretical concentration, ng/mL	Volume, mL	Volume, mL	Remove, mL	Theoretical concentration, ng/mL
10	0.1	10	0.1	0.1
20	0.1	10	0.1	0.2
250	0.1	10	0.1	2.5
500	0.1	10	0.1	5
1000	0.1	10	0.1	10
2000	0.1	10	0.1	20
3000	0.1	10	0.1	30
4000	0.1	10	0.1	40
5000	0.1	10	0.1	50

Table 1
PREPARATION OF CALIBRATION STANDARDS SOLUTIONS

Working solution for quality control			Plasma blank	Quality control
Theoretical concentration, ng/mL	Volume, mL	Volume, mL	Remove, mL	Theoretical concentration, ng/mL
10	0.1	10	0.1	0.1
30	0.1	10	0.1	0.3
1250	0.1	10	0.1	12.5
3500	0.1	10	0.1	35
5000	0.1	10	0.1	50

Table 2
PREPARATION OF QUALITY CONTROL SOLUTIONS

Mode	MRM
Ionization mode	Positive
Source	API-ESI
Drying temperature	350°C
Nebulizer pressure	60 psi
Flow rate of drying gas	11 L/min

Table 3
MASS SPECTROMETER SETTINGS

The stock solutions, the intermediate solutions and the working solutions for calibration standards and quality control samples were stored in the refrigerator at -25±10°C. The internal standard working solution was stored at 5±3°C when not in use.

The calibration and QC samples were prepared by spiking the blank plasma with an appropriate amount of alprazolam, as shown in table 1 and 2. Replicate 200 µL solutions of each calibration standards and quality control samples were stored at 25±10°C.

Human plasma extraction

Samples were extracted in the following manner: lorazepam internal standard working solution (50 µL) was added to 200 µL human plasma mixed with EDTA-K_3 anticoagulant and the solution was vortex stirred at 1200 rpm for approx. 30 s. 2 mL isobutyl-methyl ether was added and the tubes were horizontally stirred (for approx. 5 min.), then samples were centrifuged (approx. 3900 rpm for 5 min.), the organic layer was transferred to a clean glass tube and evaporated to dryness at normal pressure and 40°C. 100 µL of the mobile phase were added to the dry residue and mixed at 1.200 rpm for around 2 min. Extracts were transferred to vials prior to injection into the LC-MS/MS system.

LC-MS/MS method

All analyses were performed using the Agilent 1200 Triple Quad 6410A System. The system components included the Agilent 1200 Degasser, Agilent 1200 Binary Pump, Agilent 1200 Autosampler, Agilent 1200 Mass Selective Detector. The Agilent MassHunter software was used for system control and data acquisition.

The LC-MS/MS method for determination of alprazolam in human plasma samples described in this paper was performed using a reverse phase column Zorbax SB-CN (4.6 x 100mm, 3.5 µm). The mobile phase consisted of a mixture of 0.5% formic acid solution (pH = 3) and acetonitrile (30:70, v/v). The LC system was operated at 0.8 mL/min, using the binary pump. The column temperature was 40°C. The injection volume was 10 µL and represented no more 5% of the total sample available for injection.

The tandem mass spectrometer settings are detailed in table 3.

Alprazolam and lorazepam were monitored at m/z transitions of 309→ 281 and 321→ 275 for lorazepam, respectively, with dwell times of 100 ms and collision energy of 14, for each.

LC-MS/MS peaks were observed at approximately 2 min for both the alprazolam and lorazepam, respectively. Integration was performed using the Agilent MassHunter software associated with the mass spectrometer. The calibration curve was constructed over the range 0.1-50 ng/mL by plotting the nominal concentration of each calibrant against the peak area ratio with reference to the internal standard. The regression type used was linear with a weighting factor of 1/X applied. The concentration of alprazolam in unknown, validation and QC samples was back-calculated from the calibration curve.

Results and discussions

Validation of the alprazolam assay

The parameters usually examined in the validation process are selectivity, specificity, linearity, limit of quantification, accuracy and precision, recovery, stability [17-18].

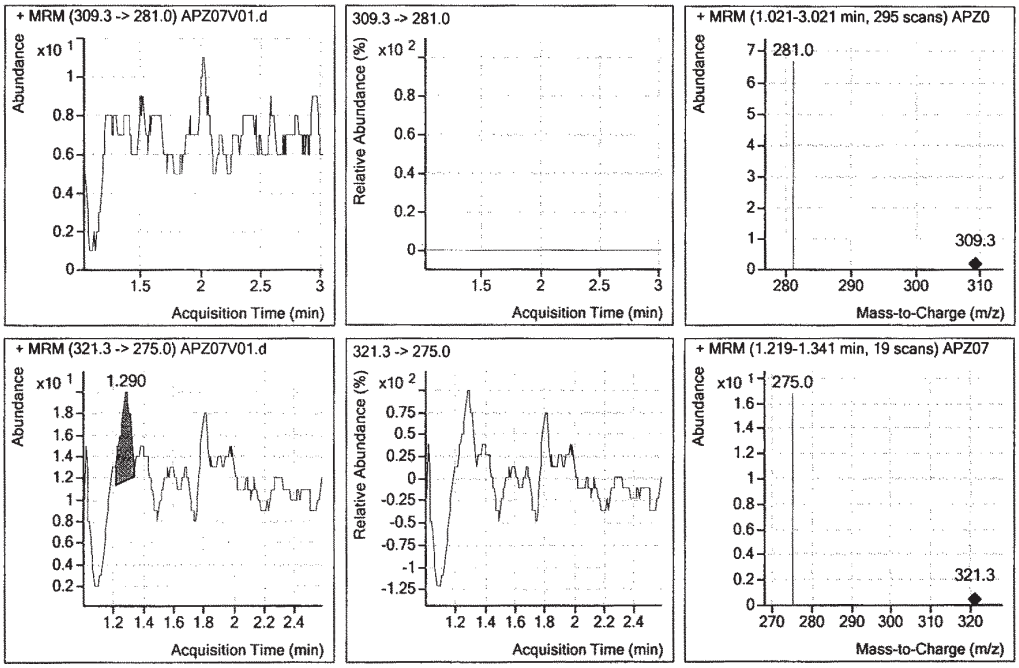

Fig. 2. Chromatogram of blank sample

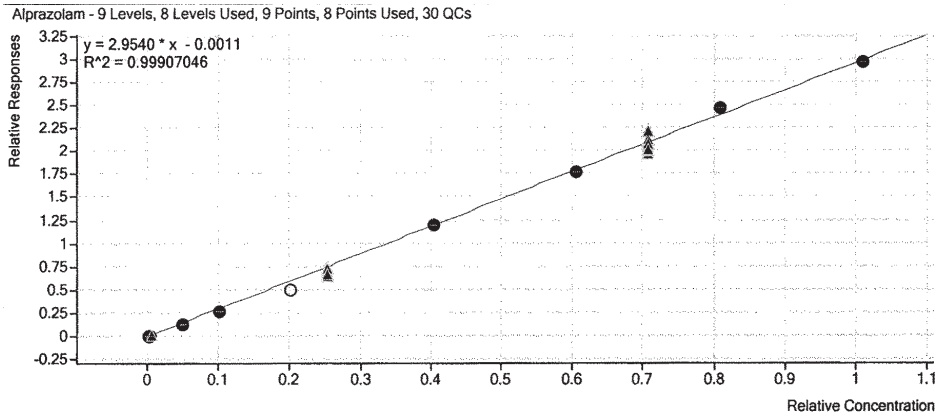

Fig. 3. Alprazolam calibration curve obtained for plasma samples

Selectivity and Specificity

The method described in this paper has been tested for possible interferences from other plasma factors. Plasma aliquots from six different sources were assessed for analysis in order to investigate the plasma components behaviour.

As it can be seen in the figure 2, no overlapping peaks were detected at the retention time of alprazolam and internal standard, 2 min and 1.6 min, respectively. No endogenous components interfering with the analyte and the internal standard were found in the chromatograms obtained from blank plasma samples.

Linearity

The linearity was investigated for alprazolam theoretical concentration levels in the range between 0.1 ng/mL and 50 ng/mL and the calibration curve was derived by plotting the peak-height ratios of the analyte and the internal standard, using linear regression analysis.

The least-square linear regression revealed that the relationship between concentration and peak-height was linear in the investigated domain, with a correlation coefficient of 0.9988, meeting the acceptance criteria ($r^2 \geq 0.990$), as it can be seen in figure 3.

Quantification limit

The lower limit of quantitation, i.e. the lowest standard level with a coefficient of variation less than 20% and a signal to noise ratio higher than or equal to 5, was 0.1 ng/mL alprazolam. The bioanalytical method proved to be sensitive, allowing a precise quantification of concentration as low as 0.1 ng/mL (fig. 4). The results are shown in table 4.

Accuracy and precision

The accuracy and precision of this method were calculated for three concentration levels of alprazolam in human plasma. Six replicate samples having alprazolam theoretical concentration levels of 0.303 ng/mL (QC1), 12.625 ng/mL (QC2) and 35.350 ng/mL (QC3) were injected into the system. Table 5 summarizes the results obtained for the intra-day parameters. The inter-day precision and accuracy were evaluated also using six aliquots for each quality control sample concentration, prepared and analyzed in six different days. The results are summarized in table 6.

Recovery

Recovery of alprazolam was evaluated by comparing analyte response of six extracted samples of low, medium and high quality control samples to those of six appropriately diluted standard solutions. Average recovery values for alprazolam were 76.632, 71.783 and 81.395% at low, medium and high quality control levels, respectively. Results are presented in table 7.

For internal standard, average internal standard response of eighteen extracted samples was compared to the average internal standard responses of eighteen appropriately diluted internal standard solutions. Mean

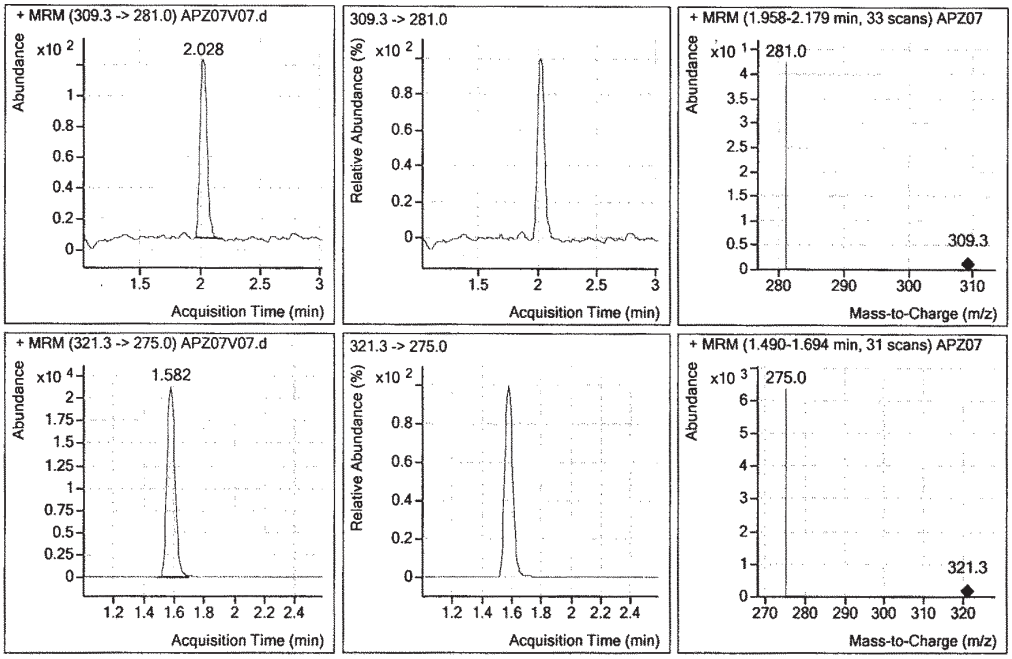

Fig. 4. Chromatogram recorded for 0.1 ng/mL alprazolam sample

	Calculated Concentration (ng/mL)	Accuracy %	Signal/Noise Ratio
Alprazolam 0.1 ng/mL	0.112	110.748	11.204
	0.147	145.452	6.567
	0.105	104.165	8.839
	0.114	112.392	8.206
	0.109	107.861	7.590
	0.116	115.225	11.204
Average	0.117	115.974	8.668
% Deviation	0.015		
% RSD	12.876		

Table 4
LOWER LIMIT OF QUANTIFICATION

No.	QC1 0.303 ng/mL		QC2 12.625 ng/mL		QC3 35.350 ng/mL	
	Found (ng/mL)	Recovery %	Found (ng/mL)	Recovery %	Found (ng/mL)	Recovery %
1	0.272	89.627	11.984	94.921	40.010	113.182
2	0.294	97.073	13.157	104.215	34.164	96.644
3	0.309	102.081	11.964	94.763	38.870	109.958
4	0.311	102.488	13.969	110.646	37.816	106.977
5	0.301	99.307	12.521	99.175	46.504	131.553
6	0.310	102.167	13.492	106.864	36.510	103.282
Average	0.299	98.791	12.848	101.764	38.979	110.266
% Deviation	0.015		0.825		4.203	
% RSD	5.022		6.421		10.783	
Overall % Deviation	1.7					
% RSD	5.0-10.7					

Table 5
EVALUATION OF PRECISION AND ACCURACY FOR ALPRAZOLAM QUALITY CONTROL SAMPLES

No.	QC1 0.303 ng/mL		QC2 12.625 ng/mL		QC3 35.350 ng/mL	
	Found (ng/mL)	Recovery %	Found (ng/mL)	Recovery %	Found (ng/mL)	Recovery %
1	0.278	91.812	11.643	92.224	30.560	86.451
2	0.294	96.981	11.333	89.767	30.296	85.703
3	0.348	114.720	10.946	86.701	32.214	91.127
4	0.271	89.538	11.652	92.296	28.954	81.906
5	0.295	97.260	10.282	81.445	32.719	92.558
6	0.295	97.489	11.169	88.471	32.484	91.894
Mean	0.297	97.965	11.171	88.482	31.205	88.273
% Deviation	0.027		0.514		1.500	
% RSD	9.108		4.602		4.808	
Overall % Deviation	0.7					
% RSD	4.6-9.1					

Table 6
EVALUATION OF INTER-DAY PRECISION AND ACCURACY FOR ALPRAZOLAM SPIKED QUALITY CONTROL SAMPLES

recovery value for the internal standard was 88.771%. Results are presented in table 8.

Stability

Room-temperature stability of analyte in human plasma was assessed by repeated determination of the analyte in six sets of QC1 (0.303 ng/mL) and QC3 (35.350 ng/mL). Each set was left at room temperature for 4 h before sample processing. There were no significant variations (less than 15% of nominal value) for alprazolam concentrations before and after storing at room temperature.

Freeze-thaw stability (-25±10°C) of analyte in human plasma was determined through recovery (%) when compared to the nominal value of QC1 and QC3 (0.303 and 35.350 ng/mL alprazolam). The test was performed in six runs. Deviation was less than 15% of the nominal value.

	Extracted peak area	Unextracted peak area	Mean Recovery (%)
QC1 (0.303 ng/mL)			
Mean	1306.878	1705.395	
S.D.	52.629	32.303	
% CV	4.027	1.894	
N	6	6	76.632
QC3 (35.350 ng/mL)			
Mean	161342.967	198222.845	
S.D.	14489.582	3844.347	
% CV	8.981	1.939	
N	6	6	81.395

Table 7
RECOVERY OF ALPRAZOLAM FROM BIOLOGICAL MATRIX

	Internal Standard Response for Extracted Sample	Internal Standard Response for unextracted Sample
Average	75404.589	84942.473
SD (±)	2427.919	1992.693
CV (%)	3.220	2.346
Concentration (ng/mL)	50.500	50.50
Mean Recovery (%)	88.771	

Table 8
RECOVERY OF INTERNAL STANDARD FROM BIOLOGICAL MATRIX

Cycle	Concentration	Nominal value (ng/mL)	Average amount (ng/mL)	Variation (%)
0	Low	0.303	0.305	0.6
	High	35.350	35.593	0.7
1	Low	0.303	0.311	2.6
	High	35.350	38.458	8.8
2	Low	0.303	0.305	0.7
	High	35.350	38.716	9.5
3	Low	0.303	0.317	4.4
	High	35.350	34.998	-1.0
4	Low	0.303	0.287	-5.2
	High	35.350	35.327	-0.1
5	Low	0.303	0.297	-2.0
	High	35.350	34.978	-1.1
6	Low	0.303	0.282	-6.9
	High	35.350	37.757	6.8

Table 9
RESULTS OF THE DETERMINATION OF ANALYTE STABILITY IN HUMAN PLASMA AT ROOM TEMPERATURE

Cycle	Concentration	Nominal value (ng/mL)	Average amount (ng/mL)	Variation (%)
0	Low	0.303	0.305	0.6
	High	35.350	35.593	0.7
1	Low	0.303	0.335	10.4
	High	35.350	32.680	-7.6
2	Low	0.303	0.287	-5.4
	High	35.350	36.831	4.1
3	Low	0.303	0.310	2.3
	High	35.350	37.861	7.1
4	Low	0.303	0.322	6.2
	High	35.350	37.534	6.1
5	Low	0.303	0.308	1.7
	High	35.350	33.263	-6.0
6	Low	0.303	0.292	-3.5
	High	35.350	32.660	-7.7

Table 10
RESULTS OF THE DETERMINATION OF ANALYTE STABILITY IN HUMAN PLASMA AFTER 3 FREEZE-THAW CYCLES AT 25±10°C

The results are summarized in table 9 and 10, respectively.

Conclusions

A new liquid chromatography-tandem mass spectrometry method has been developed and validated for the assay of alprazolam in human plasma in the concentration range 0.1-50 ng/mL. There were no interferences from endogenous plasma components or from other sources. Alprazolam and the internal standard (lorazepam) were well separated and their peaks were narrow and symmetrical. The precision and accuracy of the assay were both good. The simple sample-preparation procedure and short retention time enable determination of many samples per day.

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