Determination of Alprazolam in Rabbit Plasma by GC-MS Method

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Abstract

This paper describes a gas chromatography-mass spectrometry (GC-MS) method for determination of alprazolam in rabbit plasma. Alprazolam and internal standard (IS) medazepam were extracted from plasma by using liquid-liquid extraction method. The samples were separated by GC on a DB-5MS analytical column and determined by a quadrupole mass spectrometer detector operated under selected ion monitoring mode (SIM). Excellent linearity was found between 50 and 1000 ng/mL (r=0.998) for plasma samples. Intra-day and inter-day precisions expressed as the relative standard deviation (RSD) for the method were 1.07-2.69% and 2.42-3.98%, respectively. The mean recovery of alprazolam samples was 98.82%. The limits of detection and quantification of alprazolam were 15 and 50 ng/mL, respectively. Also, the method was successfully applied to three New Zealand white rabbits which had been given an oral tablet of 1.0 mg alprazolam.

Keywords: Alprazolam; GC-MS; Liquid-liquid extraction; Validation

Introduction

Benzodiazepine drugs are widely prescribed for their properties as anti-convulsants, anesthetics, antidepressives, hypnotics, tranquillizers and sedatives [1]. Alprazolam (Fig. 1), (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazole [4,3,-α]-[1,4] benzodiazepine) is a benzodiazepine derived from 1,4-benzodiazepines of new generation [2]. Alprazolam is mainly used to treat anxiety disorders. On a short time basis it used to palliate symptoms of anxiety or anxiety associated to symptoms of depression. Besides, alprazolam is also used to treat panic disturbances with or without agarophobia [3, 4]. Thus, extraction and identification of alprazolam in plasma is very important for forensic and clinical toxicology. However, an analysis of alprazolam in biological fluids is quite complex because the diversity of those available on the market and the fact that each drug has a particular therapeutic and toxic range.

Several methods have been reported for the determination of alprazolam including voltammetry [5], GC-MS [6-10], high performance liquid chromatography (HPLC) [11-15], LC-MS [16, 17] and LC-MS-MS [18] in pharmaceutical preparations and biological fluids.

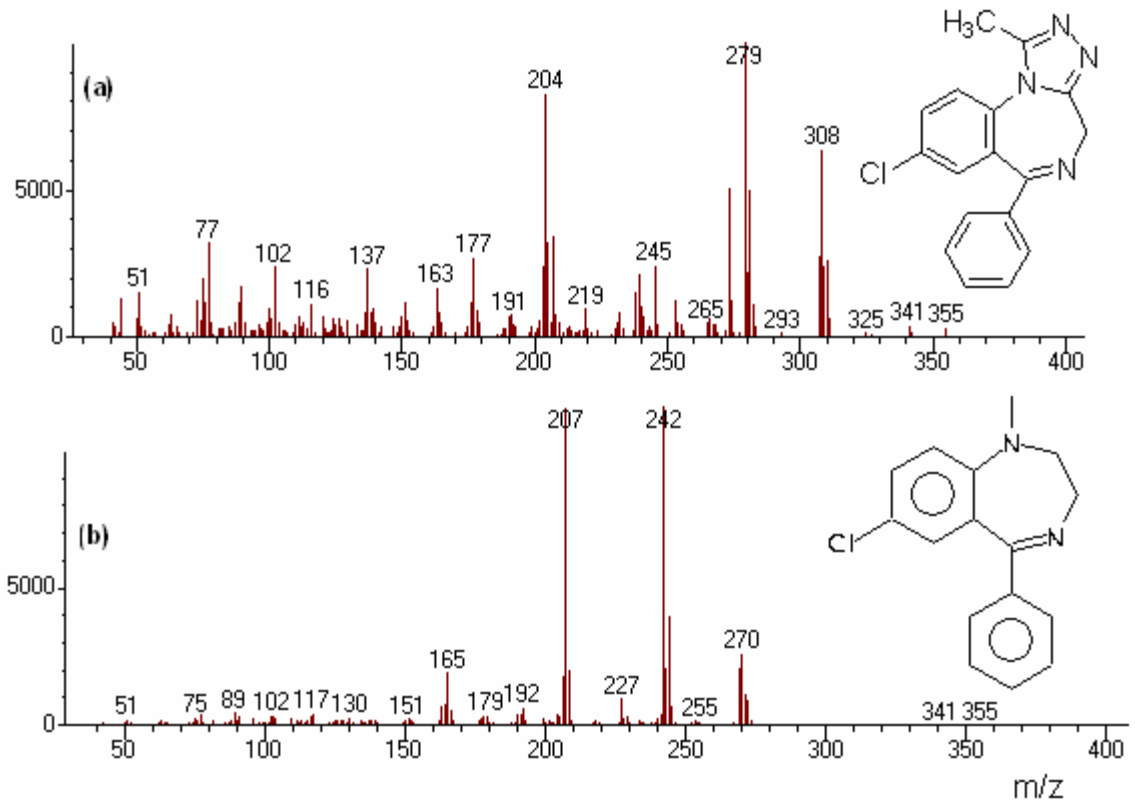

Fig. 1. Structural formula and MS spectra of alprazolam (a) and medazepam (IS) (b)

In addition, no method is reported till date for determination of alprazolam by GC-MS in rabbits which had been given alprazolam. Therefore, this report describes a simple and specific GC procedure with MS detection for determining alprazolam in rabbit plasma. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization (ICH) guidelines [19].

The advantages of present method include simple and single step extraction procedure using inexpensive chemicals and short run time. Also, this method was used to assay the alprazolam in plasma samples obtained from three rabbits which had been given an oral tablet of Xanax tablet (1.0 mg alprazolam).

Materials and Methods

Chemicals and Reagents

Alprazolam and medazepam as internal standard (IS) were obtained from Criminal Police Laboratory (Erzurum, Turkey). Chloroform and butyl chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xanax tablet containing 1.0 mg of alprazolam was obtained from pharmacy (Erzurum, Turkey).

GC-MS System

Chromatographic analysis was carried out on an Agilent 6890N gas chromatography system equipped with 5973 series mass selective detector, 7673 series autosampler and chemstation (Agilent Technologies, Palo Alto, CA). HP-5 MS column with 0.25 µm film thickness (30 m × 0.25 mm I.D., USA) was used for separation. Splitless injection was used and the carrier gas was helium at a flow rate of 1 mL/min. The injector and detector temperatures were 250 ^{0}C. The MS detector parameters were transfer line temperature 280 ^{0}C, solvent delay 3 min and electron energy 70 eV.

Preparation of Stock and Standard Solutions

The stock solution of alprazolam (1 mg/mL) was prepared and diluted with chloroform to give standard solutions of 50-1000 ng/mL (50, 100, 200, 400, 600, 800 and 1000 ng/mL). Standard calibration samples were prepared daily by spiking 1.0 mL of drug-free rabbit plasma with 0.1 mL of appropriate alprazolam standard solutions to achieve final concentrations of 50-1000 ng/mL for plasma. The working solution of IS was prepared by dissolving in chloroform to obtain a concentration of 1000 ng/mL. The quality control (QC) samples were separately prepared at the concentrations of 150, 450 and 850 ng/mL for plasma.

Extraction Procedure

Blood samples were collected into the tubes containing disodium EDTA and centrifuged at 4500 × g for 10 min. A 1 mL of the resultant plasma samples were spiked with 1 mL of alprazolam, 0.5 mL of internal standard and 1 mL Na_2CO_3 solutions were added. After vortex mixing for 5 s, 4 mL of butyl chloride was added. The mixture was vortexed for 30 s and then centrifuged at 3000×g for 3 min. The organic layer was transferred into another tube and evaporated to dryness at room temperature under nitrogen gas. The dry residue was dissolved in 1 mL of chloroform and then 1 µL sample was injected into the GC-MS system.

Rabbits

The study was conducted in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethical Committee for Medical Experimental Research and Application Centre of Ataturk University. The rabbits are male which is 3.8-4.1 kg weight. The rabbits were housed with free access to food and water, except for the final 2 h before experiment. After a single oral administration of 1.0 mg of alprazolam (Xanax tablet), 2.5 mL of blood samples were collected from the marginal ear vein at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 h time-points into EDTA collection tubes. The blood samples were centrifuged at 4000 rpm for 10 min and the plasma was taken and stored at -20 °C until analysis.

Results and Discussion

Method Development and Optimization

The method development for the assay of alprazolam was based on its chemical properties. Alprazolam is a medium polar molecule. Therefore, the capillary column coated with 5% phenyl, 95% dimethylpolysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations. Different temperature programs were investigated for GC oven. The end of this investigation, the best temperature program was selected for a good separation. The temperature programs of the GC oven was as follows: initial temperature 120 ^{0}C for 1 min and then increased to 300 ^{0}C at a rate of 50 ^{0}C/min for 6 min. The splitless injection mode was chosen. Additionally, preliminary precision and linearity studies performed during the development of the method showed that the 1 µL injection volume was reproducible and the peak response was significant at the analytical concentration chosen.

Validation of the Method

The validation was carried out by establishing specifity, linearity, intra- and inter-day precision, accuracy, recovery and sensitivity parameters according to ICH [19].

Specificity

The specificity of method was determined by checking the chromatograms obtained from blank plasma samples, and no endogenous interferences were encountered (Fig. 2). The fragment ions (m/z 279 and 242) were used for quantitation of alprazolam and IS. The retention times of alprazolam and IS were 9.3 and 7.2 min, respectively and the total run time of analysis was 10 min. Commonly prescribed drugs (carvedilol, nebivolol, atenolol, metoprolol, mexiletine, rofecoxib, diazepam, disulfiram, estradiol valerate and medroxyprogesterone acetate) were analysed for possible interference. No interference was observed under the chromatographic conditions.

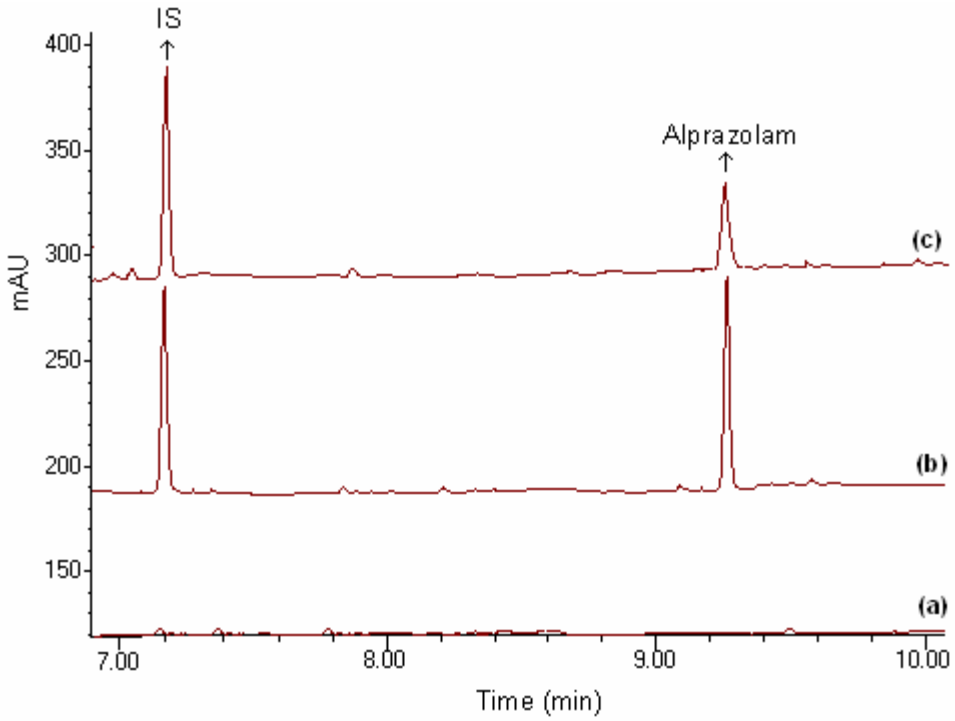

Fig. 2. Representative chromatograms of (a) drug-free rabbit plasma, (b) the rabbit plasma spiked with alprazolam (600 ng/mL) and IS (500 ng/mL), (c) the rabbit plasma obtained at 1.5 h after a single oral dose of 1.0 mg alprazolam

Linearity

Calibration curve was linear over the range 50-1000 ng/mL for plasma The regression equation was as follows:

$A = 0.2642C - 0.0146$ ($r = 0.998$) for plasma, where A is the peak area ratio ($A_{alprazolam}/A_{IS}$) and C is the concentration of alprazolam (ng/mL).

Precision and Accuracy

Intra-day and inter-day precision and accuracy were determined by replicate analysis of six sets of samples spiked with three different concentrations of alprazolam (150, 450 and 850 ng/mL) within a day or during three consecutive days. The precision was calculated from the ratio of the standard deviation to the mean (relative standard deviation, RSD). The accuracy of the method was examined by comparing the concentrations of spiked samples to the theoretical concentrations. Both values were expressed as percentage. The results of precision and accuracy were presented in Table 1.

		Concentration (ng/mL)		%RSD	%RE
Sample		Added	Found (Mean ± SD)		
Plasma		150	146.2 ± 3.938	2.69	-2.53
Intra-day		450	436.3 ± 5.252	1.2	-3.04
		850	862.3 ± 9.245	1.07	1.45
		150	139.2 ± 5.254	3.77	-7.2
Inter-day		450	458.6 ± 18.253	3.98	1.91
		850	832.6 ± 20.120	2.42	-2.05

Table 1. Intra-day and inter-day precision and accuracy of alprazolam in rabbit plasma (n=6)

The intra-day precision and accuracy were varied between 1.07 and 2.67%, and 96.9 and 101.5%, respectively. The inter-day precision and accuracy ranged from 2.42 to 3.98% and 92.8 to 101.9%, respectively. All the values of precision and accuracy including LOQ were within the specified ranges and therefore acceptable. The acceptable range of intra-day and inter-day accuracy and precision are below 15% bias or RSD. In statistical comparison ($p > 0.05$) with other methods in the literature [6, 9 and 17] the proposed method has indicated high accuracy and precision.

Limits of detection (LOD) and quantitation (LOQ)

The limit of quantification values for each sample were accepted as the lowest concentration on the calibration curves for 50 ng/mL. Under the experimental conditions, the limit of detection value was 15 ng/mL for plasma with a signal to noise ratio 3.

Recovery

It was determined that liquid-phase extraction process was necessary at the sample preparation procedure. Several solvents (butyl chloride, ethylacetate, hexane, dichloromethane, acetonitrile and butanol) were tested for the extraction. Finally, butyl chloride proved to be the most efficient in extracting alprazolam in rabbit plasma.

The recovery was determined by comparing peak area of alprazolam after extraction to that before extraction at concentrations of 150, 450 and 850 ng/mL. The mean extraction recovery of alprazolam in rabbit plasma was 98.82%. The mean relative recovery for IS at 500 ng mL^{-1} was 96.14% (n = 6). Recovery data are shown in Table 2.

		Concentration (ng/mL)		%Recovery	%RSD
Sample		Added	Found (Mean ± SD)		
Plasma		150	147.1 ± 3.901	98.07	2.65
		450	436.2 ± 6.286	96.93	1.44
		850	862.4 ± 14.282	101.46	1.66

Table 2. Recovery of alprazolam in rabbit plasma (n=6)

Alprazolam was extracted from plasma with a solid phase extraction procedure by Pongraveevongsa et al. [11], Hall et al. [13] and Quintela et al. [16]. These methods are also the most comprehensive method which can extract alprazolam in a single extraction procedure. The mean recovery is better for plasma than those of the studies reported by Pongraveevongsa et al. [11], Hall et al. [13] and Quintela et al. [16].

Stability

The stability of alprazolam in rabbit plasma was assessed by analyzing low (150 ng/mL), medium (500 ng/mL) and high (1000 ng/mL) concentration level samples after storage for different times and temperatures. The short-term temperature stability was assessed by analyzing three aliquots of each of the low, medium and high concentration samples at room temperature for 8 h. Freze-thaw stability (-20 °C in plasma) was checked through three cycles. Samples were stored at -20 °C for 24 h and then thawed unassisted at room temperature. When completed thawed, samples were refrozen for 24 h. Samples were analyzed after three freze-thaw cycles. The long-term stability was assessed after storage at -20 °C for 4 weeks. The results of the stability studies was given in Table 3 and no significant degradation of alprazolam was observed under the tested conditions.

		Recovery (Mean ± SD)		
Treatment		Plasma concentration (ng/mL)		
		150	500	1000
Three freeze-thaw cycles		96.24 ± 3.064	93.52 ± 2.932	89.24 ± 4.232
Stored at RT for 24h[a]		89.94 ± 2.842	89.27 ± 2.851	91.25 ± 3.420
Stored at -20 ^{0}C for 2 weeks		91.14 ± 2.624	92.46 ± 3.472	95.46 ± 3.726
Stored at -20 ^{0}C for 4 weeks		93.14 ± 3.241	89.72 ± 2.523	95.79 ± 4.052

[a] RT, room temperature

Application

Today, GC-MS is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples. As compared to HPLC, high-resolution capillary GC has been less frequently used [20].

The plasma samples obtained three rabbits were assayed with the validated method described above. The peaks of alprazolam and IS were completely separated from endogenous peaks with similar retention times to those of the samples used for the validation studies. Representative chromatograms of (a) drug-free rabbit plasma, (b) the rabbit plasma spiked with alprazolam (600 ng/mL) and IS (500 ng/mL), (c) the rabbit plasma obtained at 1.5 h after a single oral dose of 1.0 mg alprazolam were given in Fig. 2. There is no interference in the chromatogram of drug-free plasma.

Quintela et al. [16] have reported LC method with mass detection for the analysis of alprazolam in human plasma. The calibration curve of LC-MS method was linear for alprazolam in the range 2.0-2000 ng/mL. Intra- and inter-day precision ranged from 2.80 to 6.10 and from 1.50 to 5.70% for alprazolam, respectively. The recoveries were higher than 50.0% in all cases. The LOQ and LOD of method were found 2.0 and 1.0 ng/mL, respectively. Detection using LC-MS would be a more sensitive approach but is costly and not yet available for every laboratory.

Conclusion

In the present work, a simple and sensitive GC-MS method has been developed for the determination of alprazolam in rabbit plasma. The method was completely validated by using stability, specificity, linearity, sensitivity, accuracy and precision parameters for determination of alprazolam in rabbit plasma. Also, the extraction procedure in this study was simple. No significant interferences and matrix effect caused by endogenous compounds were observed.

To our knowledge, this is the first description of alprazolam analysis in rabbit plasma by GC-MS method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of alprazolam for clinical use.

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