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Simultaneous quantification of alprazolam, 4- and α-hydroxyalprazolam in plasma samples using liquid chromatography mass spectrometry

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Abstract

A sensitive and specific method was developed for quantification of alprazolam and its two metabolites 4-hydroxyalprazolam and α-hydroxyalprazolam in plasma. The work up procedure was solid phase extraction. Liquid chromatography–mass spectrometry (LC–MS) was used for separation, detection and quantification of the analytes. The limit of quantitation (LOQ) was 0.05 ng/mL for alprazolam and the two metabolites. The extraction recovery was more than 82% for alprazolam and its metabolites. The within- and between-assay coefficients of variation were in the range of 1.9–17.9%. The method was used for determination of the pharmacokinetics parameters of alprazolam and its two metabolites in healthy Caucasian subjects who ingested 1 mg of alprazolam.

Keywords: Liquid chromatography–mass spectrometry; Alprazolam; 4-Hydroxyalprazolam; α-Hydroxyalprazolam; Pharmacokinetics

1. Introduction

Alprazolam is a benzodiazepine, which is used for treatment of anxiety, panic disorders, depression and sleeping disorders [1]. The drug is rapidly absorbed and has a bioavailability of around 80–100% [2]. In humans, the drug is metabolized by the major drug metabolizing subfamily cytochrome P450 CYP3A to 4-hydroxyalprazolam and α-hydroxyalprazolam [3,4], which are the main metabolites in plasma. The two metabolites 4- and α-hydroxyalprazolam are pharmacologically active accounting for 20 and 60% of the activity of alprazolam [2], respectively. The drug is eliminated in urine mainly as α-hydroxyalprazolam and alprazolam [5].

The concentrations of alprazolam and its two metabolites in plasma are low after administration of therapeutic doses of the drug. To study the pharmacokinetics of alprazolam and its two metabolites one need to quantify all the three in blood samples collected under a long period of time post-drug administration. In order to do that, sensitive and selective analytical methods have to be used. A number of methods for quantification of alprazolam are reported in the literature [6–10]. Some methods for quantification of the metabolites are reported as well [5,11,12]. Few methods [11,13] for simultaneous quantification of alprazolam and the two metabolites are available among which the method reported by Smith and Kroboth [14]. In this method the volume of the sample is not defined, the concentrations of alprazolam and metabolites at which the method was validated are too high in comparison to concentrations attained post a single dose of alprazolam, furthermore the limit of quantitation of the metabolites is about the same as maximum concentration attained after a single dose of 1 mg of the drug. The LOQ reported by Jin et al. is 5 ng/mL, which is not low enough for our planned studies. Toyo'oka et al. developed a method for determination of alprazolam, estazolam, midazolam and their metabolites, in this method no validation data is available. Some methods for determination of alprazolam and one of the metabolites are available [12,15]. None of these methods really meet our

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needs to have a simple and sensitive method for simultaneous quantification of alprazaolam and its two metabolites in plasma to be used for pharmacokinetics and metabolism studies.

The aim of this study was to develop a sensitive and specific LC–MS method for determination of alprazolam, 4- and α-hydroxyalprazolam in plasma samples using solid phase extraction as a workup procedure.

2. Experimental

2.1. Conditions

2.1.1. Chemicals

Alprazolam, with a purity of 100%, was a gift from Pharmacia Upjohn (Kalamazoo, MI, USA). The metabolites (4- and α-hydroxyalprazolam with purities of 98 and 99%, respectively) were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). The internal standard (α-hydroxyalprazolam D_5) was obtained from Radian International LLC (Austin, TX, USA). All solvents used were of analytical grade.

2.1.2. Equipment

The liquid chromatograph (Agilent 1100, Agilent Technologies, Palo Alto, CA, USA) was coupled to a mass spectrometer (Agilent 1100 MSD, Agilent Technologies) with an atmospheric pressure ionization (API) electrospray (ES) interface and was used in positive-ionization mode with the following spray chamber settings: nebulizer pressure 137.9 kPa, capillary voltage 2000 V, drying gas temperature 350 °C, drying gas flow rate 10 L/min. Selected ion monitoring was chosen, and the following *m/z* values were monitored: 309 (protonated molecular ion of alprazolam), 325 (protonated 4- and α-hydroxyalprazolam), and 330 (protonated molecular ion of α-hydroxyalprazolam D_5, IS). Fragmentor voltage was set at 130 V. Chemstation software (Agilent Technologies) was used for data registration and calibration.

2.1.3. Sample preparation

To develop and evaluate the method, plasma samples were spiked with alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam and the internal standard (α-hydroxyalprazolam D_5). After validation the method was used to analyze samples from healthy Caucasian subjects who received a single oral dose of 1 mg of alprazolam (Xanor®, Pharmacia Upjohn, Stockholm, Sweden) (Wennerholm et al., 2004, manuscript). Blood samples were collected into heparinized vacuum tubes before drug intake and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 24, 48 and 72 h thereafter. The samples were centrifuged after 15 min at 1500 × *g* for 10 min; plasma was separated and stored at −70 °C until analysis.

All stock solutions and samples containing 4-hydroxyalprazolam were stored at −70 °C until analysis. The samples were left no more than 5 h at room temperature during sample workup due to the instability of 4-hydroxyalprazolam.

2.1.4. Column liquid chromatography

Column: A Luna C18 column (100 mm × 2.0 mm column, 3 μm particle size; Phenomenex, Torrance, CA, USA) was used. The column was kept at 40 °C.

Mobile phase: A mobile phase of 25 mmol/L formic acid was used with a linear gradient from 36 to 42% acetonitrile during the first 4.5 min. Thereafter, 70% acetonitrile was used for 1 min, followed by a mobile phase with 36% acetonitrile for 4.5 min. The flow rate was 0.3 mL/min. The total run time was 10 min.

2.1.5. Analytical procedure

The plasma was centrifuged at 3500 × *g* for 5 min to remove particles from the plasma. A total of 980 μL plasma, 20 μL internal standard (500 ng/mL) and 1.0 mL sodium hydroxide (0.5 mol/L) were mixed and the mixture was passed through an Oasis HLB 1 mL 30 mg extraction cartridge (Waters, Milford, MA, USA) after conditioning with 1 mL methanol and 1 mL water. The cartridge was washed with 1 mL water, and then with 1 mL 20% acetonitrile in water. Alprazolam and metabolites were eluted with 500 μL of 100% acetonitrile. Samples were dried in a vacuum centrifuge and subsequently dissolved in 50 μL 20% acetonitrile in water. Aliquots of 15 μL were injected into the LC–MS.

2.1.6. Standard curves

Standard curves for validation were prepared by adding known amounts of alprazolam and metabolites to blank plasma. All calibration curves consisted of one blank sample and five calibration points. The concentration ranges of the standard curves for alprazolam, 4- and α-hydroxyalprazolam were 0.025–40 ng/mL. Standard curves in water were compared to the standard curves in blank plasma. The resulting peak area ratios were plotted against the concentrations.

2.1.7. Recovery

To document the recovery, standard alprazolam and metabolites were added to plasma ($n = 8$). Samples were prepared according to the procedure above, and the peak areas were compared to those of directly injected standards.

2.1.8. Within- and between-assay imprecision and accuracy

To evaluate within- and between-assay imprecision and accuracy known amounts of alprazolam, metabolites and internal standard were added to plasma ($n = 8$). Three different concentrations of alprazolam and its metabolites were used as shown in Table 2. Samples were prepared according to the procedure above.

2.1.9. Stability

The stability of alprazolam, 4- and α-hydroxyalprazolam in plasma was studied at room temperature, −20 and −70 °C. The effect of pH on the stability of the analytes was also investigated by comparing pH 7 to 2.7 (formic acid buffer) at the above-mentioned temperatures.

3. Results and discussion

Peaks of alprazolam, the internal standard, 4- and α-hydroxyalprazolam are well separated from each other (Fig. 1). The ion chromatograms of blank plasma, spiked plasma and plasma from a volunteer taking a single 1 mg oral dose of alprazolam are shown in Fig. 1C. Retention times were approximately 2.8, 3.1, 3.1 and 4.1 min for 4-hydroxyalprazolam, α-hydroxyalprazolam, IS and alprazolam, respectively.

The limit of quantification was taken as the lowest concentration used for evaluation of the method, which was 0.05 ng/mL for alprazolam, 4- and α-hydroxyalprazolam. Crouch et al. have reported the same lower limit of quantitation of alprazolam and α-hydroxyalprazolam in their study, but strange enough no validation of the method at this concentration was performed. Besides the 4-hydroxyalprazolam was not determined in this method [12]. Our method reports the lowest limit of quantitation of all methods available.

The ranges for the standard curves used for validation of within and between assay imprecision and accuracy were 2.5–40 ng/mL for high and middle concentrations and 0.05–2 ng/mL for low concentrations. All the standard curves were linear within the concentration range of interest and the correlation coefficient was more than 0.99 in all the runs. Standard curves in water were shown to be consistent with the standard curves in blank plasma and hence standard curves in water were used for the analysis of volunteer samples.

Extraction recovery of alprazolam and its two metabolites is shown in Table 1. Recovery in plasma was more than 82% for all substances at three different concentration levels; high (10 ng/mL alprazolam and 1.0 ng/mL metabolites), middle (5.0 ng/mL alprazolam and 0.5 ng/mL metabolites) and low (0.05 ng/mL all substances).

Table 1
Extraction recovery

Analyte ($n = 8$)	Concentration (ng/mL)	Recovery (%)	CV (%)
	10	91	2
Alprazolam	5	100	3
	0.05	83	14
4-Hydroxyalprazolam	10	89	8
	5	87	3
	0.05	100	18
α-Hydroxyalprazolam	10	99	3
	5	102	2
	0.05	100	8

The within and between assay coefficients of variation for the three analytes varies between 1.9 and 17.8% as shown in Table 2. We used low concentrations of alprazolam and its two metabolites for method validation since this is the case when using clinically relevant doses of alprazolam. The method was developed to be used for pharmacokinetics and metabolism studies and for such purposes these levels of variation are acceptable.

Plasma concentration versus time curves for alprazolam, 4- and α-hydroxyalprazolam from a representative subject are shown in Fig. 2. All samples from the same individual were analyzed in duplicate on the same day. Using this method we could quantify alprazolam, 4- and α-hydroxyalprazolam in samples collected from all the subjects up to 72, 48 and 24 h post-drug administration, respectively. In some individuals 4- and α-hydroxyalprazolam could be quantified in samples collected up to 72 and 48 h post-alprazolam administration, respectively.

At room temperature, the levels of all substances were found to be unchanged for 6 h in plasma. At −20 and −70 °C, 4-hydroxyalprazolam was stable for 3 and 7 months in plasma, respectively. No change in concentration of the other substances was observed after 5 and 10 months in plasma, respectively. The 4-hydroxyalprazolam was stable in water solution at room temperature for 5 h in contrast to the other substances that were stable for 24 h. The 4-hydroxyalprazolam was stable at −20 °C in water solution for 4 months and the other substances for 12 months. In acidic solution, 4-hydroxyalprazolam degraded rapidly in room and refrigerator temperatures and slower at −20 °C. The other substances were stable in the acidic buffer at all temperatures. Alprazolam and α-hydroxyalprazolam in water solution were stable throughout three complete freeze-and-thaw cycles. There was a tendency for degradation of 4-hydroxyalprazolam after the freeze-and-thaw cycles. These results indicate that stock solutions should be prepared in water (neutral pH) and samples containing alprazolam and its two metabolites should be kept in −70 °C if analysis will be performed in more than 3 months after sampling.

Liquid chromatography–mass spectrometry is a very powerful technique because of the high sensitivity and specificity of the mass spectrometric detection. The technique is gaining more interest in analysis of biological samples since most of the time the target concentrations are very low and high sensitivity is required. Nevertheless, some problems are associated with using this technique such as ion suppression effects, which is the effect of sample matrix on the ionization process. Different methods to minimize this effect have been published among which the use of a selective extraction procedure for the sample clean up [16] and more chromatographic retention of the analytes [17]. Since we used an extensive work up procedure for the extraction of the alprazolam and its two metabolites and the analytes were retained in the chromatographic column for an adequate time, no ion suppression effects were observed.

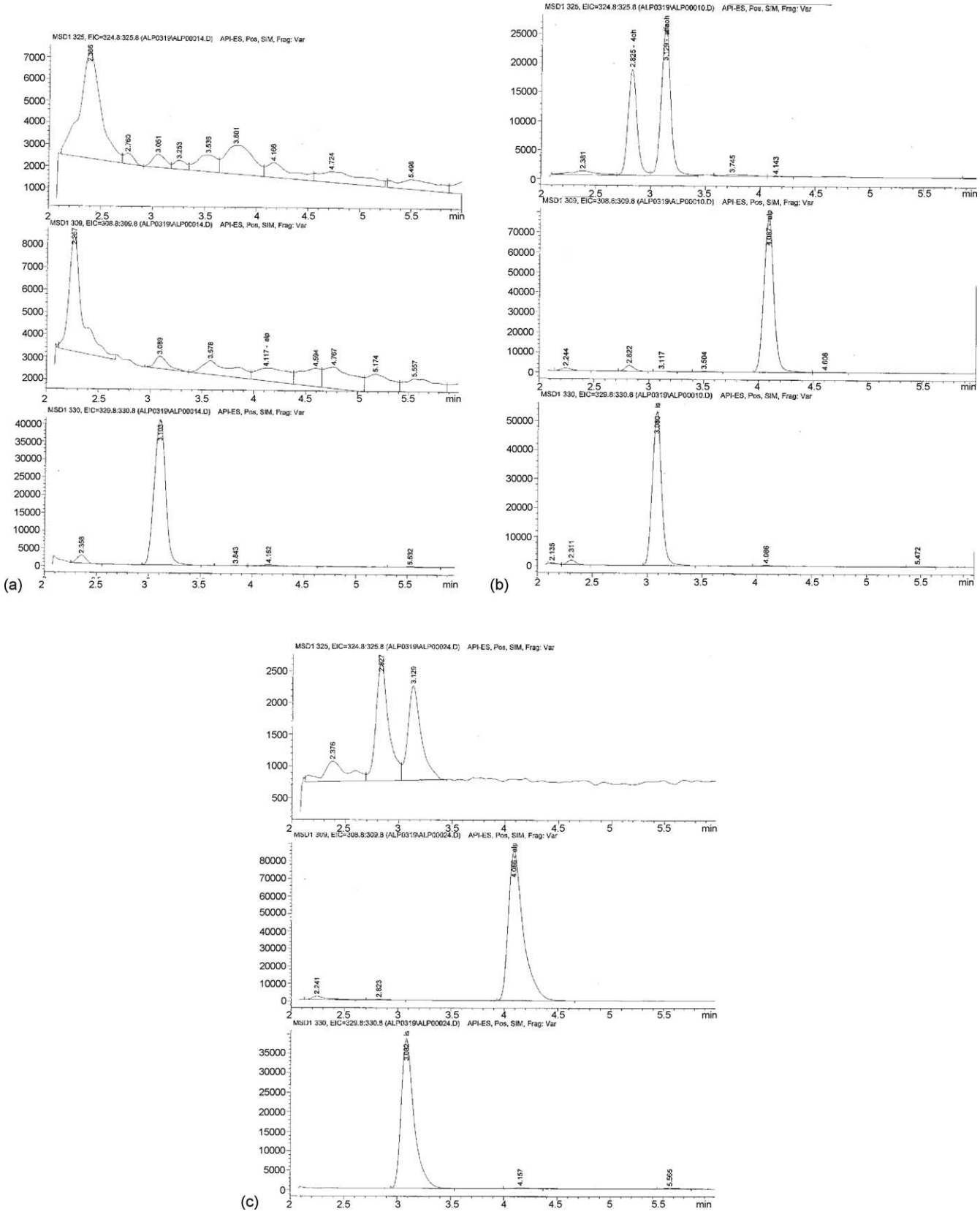

Fig. 1. Ion chromatograms of alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam and IS in (A) blank plasma; (B) plasma spiked with 10, 5, 5 and 10 ng/mL of alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam and IS, respectively. (C) Plasma collected from a healthy subject 2 h after administration of 1 mg oral dose of alprazolam containing 14.3, 0.59, 0.32 and 10 ng/mL of alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam and IS, respectively. The retention time for 4-hydroxyalprazolam, α-hydroxyalprazolam, alprazolam and the IS are 2.8, 3.1, 4.1 and 3.1 min, respectively.

Table 2
Within- and between-assay imprecision and accuracy in plasma

Substance	Nominal concentration (ng/mL)	Found concentration (ng/mL)	CV (%)	Nominal concentration (ng/mL)	Found concentration (ng/mL)	CV (%)	Nominal concentration (ng/mL)	Found concentration (ng/mL)	CV (%)
Within-assay ($n = 9$)									
Alprazolam	10.0	10.3	1.9	1.00	1.06	4.6	0.050	0.046	16.8
4-Hydroxyalprazolam	5.0	5.5	5.6	0.10	0.09	12.6	0.050	0.051	9.2
α-Hydroxyalprazolam	5.0	5.2	5.6	0.10	0.09	3.8	0.050	0.050	12.1
Between-assay ($n = 6$)									
Alprazolam	10.0	11.0	10.3	1.00	1.03	12.4	0.050	0.052	17.9
4-Hydroxyalprazolam	5.0	4.5	9.0	0.10	0.09	10.2	0.050	0.048	9.8
α-Hydroxyalprazolam	5.0	5.3	10.1	0.10	0.09	12.9	0.050	0.050	10.8

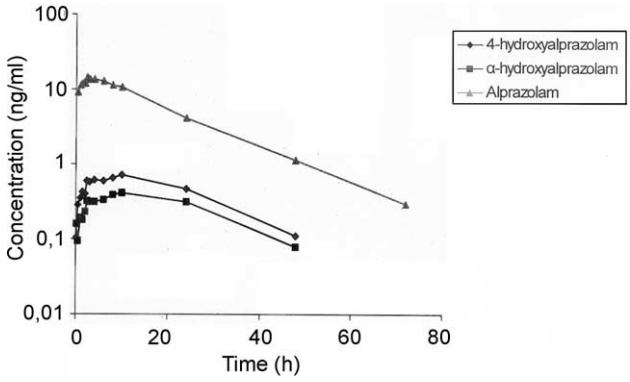

Fig. 2. Plasma concentration vs. time curves of alprazolam, 4- and α-hydroxyalprazolam after oral administration of 1 mg alprazolam from a representative subject.

In conclusion, the method described in this report is sensitive and specific. It includes thorough validation for alprazolam and its two metabolites unlike the other published methods. Using this method we can analyze samples collected in pharmacokinetics and metabolism studies. Due to the instability of 4-hydroxyalprazolam care should be taken in preparation of stock solutions and storage of samples.

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