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Determination of alprazolam and its major metabolites in serum microsamples by high-performance liquid chromatography and its application to pharmacokinetics in rats

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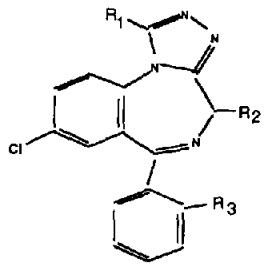
Abstract

A high-performance liquid chromatographic method using a single-solvent extraction step is described for quantitating alprazolam and its metabolites in rat serum microsamples (50 μl). The separation used a 2-mm I.D. reversed-phase Ultrasphere C_{18} column with a mobile phase of methanol–acetonitrile–sodium acetate buffer. By decreasing the methanol content in the mobile phase, triazolam and its metabolites can be extracted and separated using the same method. The detection limit was 5 ng/ml for all the compounds using an ultraviolet detector at 230 nm. The method showed the effect of aging on alprazolam pharmacokinetics following 0.8 mg/kg intravenous bolus alprazolam administration.

1. Introduction

Alprazolam, a 1,4-triazolobenzodiazepine, is the most commonly prescribed benzodiazepine in the United States. It is not only used as an anxiolytic [1,2], but is also efficient in treating panic disorder [3,4], and possibly depression [5]. Two of its major active metabolites, α-hydroxyalprazolam and 4-hydroxyalprazolam, account for 15% and less than 1% of the urinary excretion of [^{14}C]alprazolam, respectively. The two metabolites are approximately 60% and 20% as potent as alprazolam, respectively [6,7]. Structures of the compounds are shown in Fig. 1.

Electron-capture gas chromatography [8], and reversed-phase high-performance liquid chroma-



Alprazolam	$R_1 = CH_3$	$R_2 = H$	$R_3 = H$
α- Hydroxy alprazolam	$R_1 = CH_2OH$	$R_2 = H$	$R_3 = H$
4- Hydroxy alprazolam	$R_1 = CH_3$	$R_2 = CH_2OH$	$R_3 = H$
Triazolam	$R_1 = CH_3$	$R_2 = H$	$R_3 = Cl$
α- Hydroxy triazolam	$R_1 = CH_2OH$	$R_2 = H$	$R_3 = Cl$
4- Hydroxy triazolam	$R_1 = CH_3$	$R_2 = CH_2OH$	$R_3 = Cl$

Fig. 1. Structures of alprazolam, triazolam and their metabolites.

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tography (HPLC) with ultraviolet detection [9–11], have been used to determine alprazolam and its metabolites in biological samples. Other assays are available for the detection of alprazolam, for example, thin-layer chromatography [12] and immunoassay [13]. Of these methods HPLC is the most attractive and versatile technique for the determination of drugs in biological samples since it has the possibility to adjust the selectivity of the resolution over a wide range. Moreover, owing to its milder working conditions, HPLC is the most suitable technique for the analysis of thermally labile compounds.

In this paper, we report a simple HPLC method capable to determine alprazolam and its metabolites in small samples (50 μl). Sample size is critical when the animal species used is small, especially when repeated blood sampling is necessary to trace the temporal changes in drug levels in individual animals. The method described could be used to evaluate the effect of age on alprazolam pharmacokinetics in rats after an intravenous bolus dose of alprazolam (0.8 mg/kg). Moreover, by simply decreasing the methanol content in the mobile phase, the triazolobenzodiazepine triazolam, an oral hypnotic agent with a short half-life, and two of its metabolites, α-hydroxytriazolam and 4-hydroxytriazolam, which are closely related to alprazolam in chemical structure as shown in Fig. 1 [14], can also be quantitated with this method.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Waters Associates Model 510 pump (Milford, MA, USA), a 7725 Rheodyne injector with a 20-μl loop (Cotati, CA, USA), and a 785A programmable absorbance UV detector, operated at 230 nm (0.005 AUFS) (Applied Biosystems Instruments, Foster City, CA, USA). The separation was performed on a Beckman Ultrasphere C_{18} column (5 μm particle size, 150 × 2 mm I.D.) (San Ramon, CA, USA) with a 2-μm precolumn filter (Rheodyne). The data were collected using a PE Nelson 900 series interface and Turbochrom 3 software (Norwalk, CT, USA) and an IBM-type 386 microcomputer workstation.

2.2. Reagents and standards

Alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam, triazolam, α-hydroxytriazolam and 4-hydroxytriazolam were supplied by Upjohn Laboratories (Kalamazoo, MI, USA). Demoxepam was obtained from Hoffmann-La Roche (Nutley, NJ, USA). HPLC-grade methanol, acetonitrile and sodium acetate were purchased from Fisher Scientific (Springfield, NJ, USA), and diethyl ether from Aldrich (Milwaukee, WI, USA). Methoxyflurane (Metofane) was purchased from Pitman-Moore (Mundelein, IL, USA). The 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0) was prepared by the method of de Silva and Puglisi [15]. All other chemicals were reagent grade.

Alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam, triazolam, α-hydroxytriazolam, 4-hydroxytriazolam, and demoxepam were dissolved in methanol individually to make 1 mg/ml stock solutions which were then diluted to 10 μg/ml with nanopure water. These aqueous drug solutions were further diluted to the desired concentrations: 1.0, 0.5 and 0.05 μg/ml with blank serum. The internal standard, demoxepam, was diluted to a final concentration of 0.5 μg/ml. Stock solutions were freshly prepared monthly. All the stock solutions and working standards were stored at −20°C.

The HPLC analyses were performed using an isocratic mobile phase consisting of methanol–acetonitrile–43 m*M* sodium acetate in water at pH 2.4 (45:8:47, v/v/v). By decreasing the methanol content from 45% to 40% and adding 5% water to this mobile phase, triazolam and metabolites can be well resolved. Mobile phases were degassed by sonicaton after mixing for 15 min in a cold water bath. The flow-rate was set at 0.3 ml/min with a pressure of 140 bar (2000 psi).

2.3. Sample preparation

Standards and serum samples were prepared as previously described [16]. A 50-μl working serum standard and 25 μl of internal standard (demoxepam, 0.5 μg/ml) were added to a 15-ml conical centrifuge tube. Borate buffer (1 *M*, pH 9.0, 100 μl) was added and the solution was mixed well. Diethyl ether (2 ml) was added and the sample mixture was vortex-mixed for 40 s and centrifuged for 5 min at 1100 *g*. The diethyl ether layer was carefully transferred to a 5-ml conical centrifuge tube and evaporated in an evaporator (Pierce, Rockford, IL, USA) at 40°C under nitrogen. The residue was resuspended in 50 μl of the mobile phase and injected onto the column. Samples for serum drug analysis were prepared identically except that standards were not added.

2.4. Extraction recovery

The assay recoveries for alprazolam, triazolam and their metabolites were assessed at concentrations of 0.05, 0.5 and 1.0 μg/ml. Nine replicates of each concentration, containing the two compounds for alprazolam and 3 compounds for triazolam, extracted according to the method described above, were injected onto the column. Nine replicates of each concentration were computed using the following equation:

$$\text{Recovery} = (\text{peak height extract})/(\text{mean peak height direct injection}) \times 100\%$$

2.5. Alprazolam administration and serum sampling

Alprazolam (5 mg) was dissolved in 25 μl 1.2 *M* HCl and diluted to 1 mg/ml with sterile saline. Two adult male, Holtzman albino rats held to 80% of their normal, adult starting weight were used (body weight and age: rat B6, 308 g, 12 months; rat E2, 310 g, 4 months). Rats were anesthetized using methoxyflurane by the inhalation route. Right jugular vein cannulation was performed. Animals were allowed to recover for two days and then received 0.8 mg/kg alprazolam by intravenous (i.v.) bolus injection into the jugular vein cannula. Blood samples (100 μl) from the jugular catheter were obtained at 2, 4, 6, 8, 12, 16, 20, 25, 30 and 40 min. Blood samples were collected, centrifuged for 10 min at 13 700 *g* and stored frozen until analysis. The experimental protocol was reviewed and approved by the Rutgers University Animal Care and Facilities Committee.

2.6. Pharmacokinetic analysis

After i.v. alprazolam administration, a biexponential function of the following form was fitted to the experimental data.

$$C_t = A\,e^{-\alpha t} + B\,e^{-\beta t}$$

$$Cl = \text{Dose}/\text{AUC}$$

$$V_1 = \text{Dose}/(A + B)$$

$$V_2 = \text{Dose}/B$$

where, C_t is the concentration in serum at any time t, α and β are the hybrid rate coefficients for the distribution and elimination phases, A and B are the preexponential coefficients, Cl is the clearance and V_1 and V_2 are the distribution and the terminal volumes of distribution, respectively. The area under the curve (AUC) was calculated by the trapezoidal method.

3. Results

3.1. Method evaluation

Fig. 2 shows chromatograms of (A) a serum blank with no interfering peaks, (B) a spiked serum sample containing a working standard and internal standard which was extracted by the liquid–liquid extraction procedure, and (C) a representative rat serum sample (50 μl) obtained 15 min after p.o. administration of alprazolam (1.6 mg/kg). Triazolam and two of its metabolites, α-hydroxytriazolam and 4-hydroxytriazolam, can be separated and quantitated by

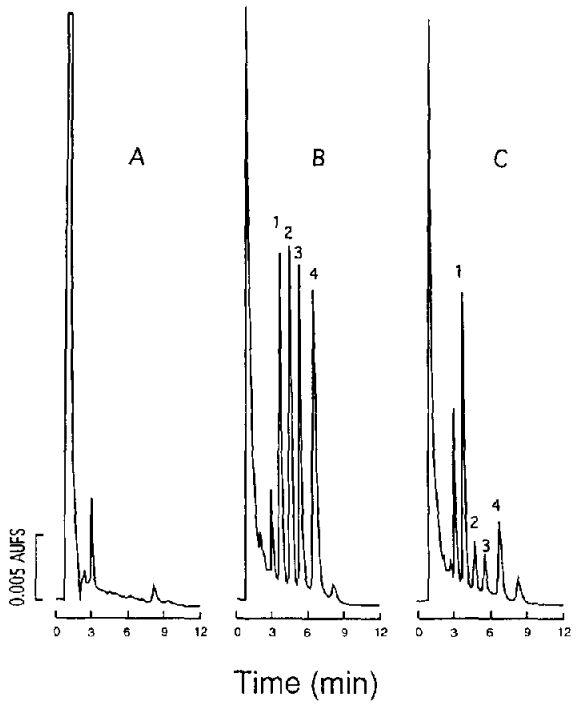

Fig. 2. Chromatograms of (A) rat serum blank, (B) rat serum containing 0.5 μg/ml 4-hydroxyalprazolam, α-hydroxyalprazolam and alprazolam, (C) a 50-μl rat serum sample obtained 15 min after administration of an 1.5 mg/kg alprazolam oral dose. Peaks: 1 = demoxepam; 2 = 4-hydroxyalprazolam; 3 = α-hydroxyalprazolam; 4 = alprazolam.

decreasing the methanol content in the first mobile phase to 40% as described in the Experimental section.

Within-day and between-day precisions were established for three different concentrations (0.05, 0.5 and 1.0 μg/ml) by adding these compounds to blank serum. The coefficients of variation (C.V.) for alprazolam and its metabolite ranged from 1.92 to 6.87% for within-day and 2.43 to 9.04% for between-day (Table 1). For triazolam and its two metabolites, the C.V. ranged from 1.70 to 5.51% for within-day and 1.93 to 6.94% for between-day.

Calibration curves for alprazolam, triazolam and their metabolites are linear within the range (0.05–1.0 μg/ml) examined. For each of the six regression lines, the correlation coefficients are less than 0.998. The coefficients of variation of the slopes ($n = 6$) of the regression lines ranged from 1.6 to 5.05% with intercepts all close to zero (Table 2). The detection limit was 0.1 ng (5 ng/ml) for all the compounds, with a signal-to-noise ratio of 4.

The recoveries (mean ± S.D.) for alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam and demoxepam were 83.92 ± 7.4, 79.64 ± 6.6, 82.59 ± 6.5 and 85.86 ± 4.0%, respectively. For triazolam, α-hydroxytriazolam and 4-hydroxytriazolam recoveries were 93.08 ± 8.3, 89.45 ± 9.9 and 82.51 ± 7.8, respectively. All the recoveries were calculated at three concentrations (0.05, 0.5 and 1.0 μg/ml) for these six compounds.

To determine the specificity of the method by using the mobile phase for alprazolam and its metabolites, Table 3 lists the relative retention times (k') of other benzodiazepines and drugs studied. Alprazolam and triazolam, as well as α-hydroxytriazolam and 4-hydroxytriazolam, partially overlapped as indicated by their k' values. There was also interference between oxazepam and alprazolam. Oxazepam and triazolam, as well as clonazepam, 4-hydroxyalprazolam and α-hydroxytriazolam, were partially resolved.

3.2. Pharmacokinetic results

Fig. 3 and Table 4 show the age-dependent pharmacokinetics for two rats, B6 and E2, after a 0.8 mg/kg i.v. administration of alprazolam. The serum alprazolam concentration showed a sharp biexponential decline in E2, who was 8 months younger than B6. However, 4-hydroxyalprazolam and α-hydroxyalprazolam were not detected after alprazolam administration by this route. The clearance and elimination half-life values were 3.7 and 3.9 times higher for E2 than for B6, respectively, but there was less variation in the values of α and V_1.

4. Discussion

By using an accurate and precise single-solvent extraction step the present HPLC method for determining alprazolam, triazolam and their me-

Table 1
Precision data for alprazolam, triazolam and their metabolites in serum

Compound	Within-day ($n = 6$)		Between-day ($n = 6$)	
	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)
Alprazolam	0.0493 ± 0.0019	3.81	0.0507 ± 0.0017	3.38
	0.4958 ± 0.0136	2.74	0.5003 ± 0.0127	2.54
	0.9964 ± 0.0254	2.55	1.0096 ± 0.0374	3.71
4-Hydroxyalprazolam	0.0495 ± 0.0034	6.87	0.0498 ± 0.0045	9.04
	0.5006 ± 0.0121	2.41	0.4997 ± 0.0364	7.28
	0.9993 ± 0.0192	1.92	1.0025 ± 0.0250	2.49
α-Hydroxyalprazolam	0.0497 ± 0.0016	3.20	0.0496 ± 0.0018	3.63
	0.4959 ± 0.0146	2.94	0.4988 ± 0.0121	2.43
	0.9940 ± 0.0260	2.62	1.0105 ± 0.0291	2.88
Triazolam	0.0509 ± 0.0026	5.11	0.0506 ± 0.0022	4.40
	0.5027 ± 0.0126	2.51	0.5008 ± 0.0242	4.84
	1.0066 ± 0.0183	1.82	1.0005 ± 0.0193	1.93
α-Hydroxytriazolam	0.0501 ± 0.0027	5.39	0.0506 ± 0.0035	6.94
	0.5034 ± 0.0111	2.21	0.5018 ± 0.0207	4.12
	1.0037 ± 0.0171	1.70	1.0022 ± 0.0201	2.01
4-Hydroxytriazolam	0.0508 ± 0.0028	5.51	0.0507 ± 0.0020	4.02
	0.5057 ± 0.0172	3.40	0.5022 ± 0.0255	5.08
	1.0043 ± 0.0228	2.27	1.0051 ± 0.0209	2.08

tabolites in serum microsamples is sensitive and rapid (Table 1 and Fig. 2). This microsample method provides a valuable technique to study the pharmacokinetics of these compounds in small animals using within-subject designs that prevent the disturbance of normal hemodynamics caused by excess blood loss. After exposure to light and room temperature, 4-hydroxyalprazolam breaks down to unknown products which may interfere with analyses [10]. When 4-hydroxyalprazolam was exposed to acidic pH for 30 min, an unknown breakdown peak appeared, which was eluted much earlier than 4-hydroxyalprazolam. The final pH of the

Table 2
Mean of six calibration equations for alprazolam, triazolam and their metabolites over the concentration ranges 0.05–1.0 μg/ml

Compound	Equation	Correlation coefficient	C.V. of slope (%)
Alprazolam	$y = 1.0119(\pm 0.0410)x - 0.0034(\pm 0.0091)$	0.999	4.05
4-Hydroxyalprazolam	$y = 1.0223(\pm 0.0247)x - 0.0057(\pm 0.0085)$	0.998	2.42
α-Hydroxyalprazolam	$y = 1.0117(\pm 0.0301)x - 0.0031(\pm 0.0063)$	0.999	2.97
Triazolam	$y = 1.0034(\pm 0.0161)x - 0.0008(\pm 0.0054)$	0.999	1.60
α-Hydroxytriazolam	$y = 1.0038(\pm 0.0160)x - 0.0005(\pm 0.0038)$	0.999	1.60
4-Hydroxytriazolam	$y = 1.0034(\pm 0.0224)x - 0.0019(\pm 0.0048)$	0.999	2.23

y = peak-height ratio (compound/internal standard), x = concentration of each compoud.

Table 3
Relative retention times (k') of other drugs tested for interference in mobile phase 1

Compound	k'	Compound	k'
Cocaine	< 2.0	α-Hydroxytriazolam	3.95
Norcocaine	< 2.0	4-Hydroxytriazolam	4.43
Cocaethylene	< 2.0	α-Hydroxyalprazolam	4.47
Buspirone	< 2.0	Hexabarbital	4.97
Flumazenil	< 2.0	Alprazolam	5.75
Chlordeazepoxide	< 2.0	Oxazepam	5.98
Midazolam	< 2.0	Triazolam	6.05
Phenobarbital	2.03	Temazepam	7.00
Flurazepam	2.41	N-1-Desalkyflurazepam	7.61
Demoxepam	2.73	Diazepam	10.69
4-Hydroxyalprazolam	3.63	Chlorpromazine	13.61
Clonazepam	3.78	Amphetamine	N.D.

N.D. = Peak not observed within 30 min.

mobile phase after mixing of all components was *ca.* 3.5. There was no early breakdown product peak observed. Furthermore, since all samples and standards were stored at −20°C instability of 4-hydroxyalprazolam was not a problem in the present study.

The α-hydroxytriazolam and 4-hydroxytriazolam peaks partially overlapped when mobile phase is used for the separation of alprazolam and its metabolites (Table 3). Although alprazolam and triazolam had close k' values in this mobile phase, it is unlikely that these two drugs will be co-administered. However, by decreasing the methanol content to 40%, triazolam and the metabolites were completely resolved with k' values of 4.12, 5.86, 6.77 and 9.32 for demoxepam, α-hydroxytriazolam, 4-hydroxytriazolam and triazolam, respectively.

Aging leads to an increase in the pharmacological effects of a variety of drugs in animals [17,18] and humans [19,20]. It was evident that age played an important role in alprazolam pharmacokinetics after i.v. bolus administration in male Holtzman albino food-limited rats (Fig. 3 and Table 4). In humans, the half-life of al-

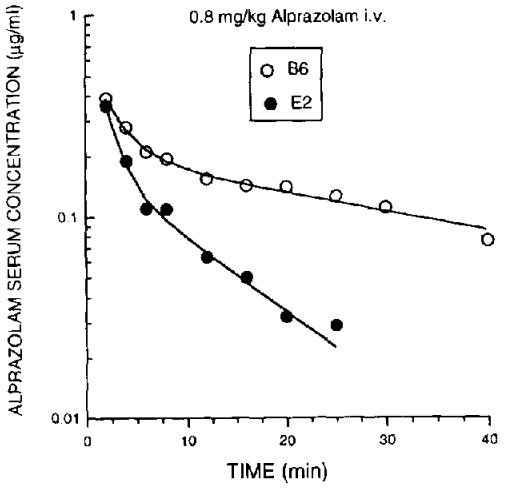

Fig. 3. Serum alprazolam concentration–time profiles for B6 and E2 following administration of an i.v. bolus of 0.8 mg/kg alprazolam.

Table 4
Pharmacokinetic parameters in young (E2) and old (B6) rat following i.v. bolus administration of 0.8 mg/kg alprazolam

Pharmacokinetic parameters	B6	E2
α (/min)	0.416	0.664
β (/min)	0.023	0.089
$AUC_{(0-\infty)}$ (μg/ml min)	9.320	2.518
$T_{1/2}(\alpha)$ (min)	1.67	1.04
$T_{1/2}(\beta)$ (min)	30.13	7.79
Cl (l/min/kg)	0.086	0.318
V_1 (l/kg)	1.24	0.85
V_2 (l/kg)	3.92	4.42
A (μg/ml)	0.442	0.759
B (μg/ml)	0.204	0.181

prazolam is prolonged and clearance reduced in elderly as opposed to young men, whereas neither clearance nor half-life is significantly altered by age in women following a single oral dose of alprazolam [21]. The present method enabled us to trace the distribution and elimination phases of alprazolam in rats by using a two-compartment model. The terminal half-life of alprazolam for rat B6 was 30.13 min, which is similar to the value of 23.52 min found in rats by Banks *et al.* [12].

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