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Note

Alprazolam, α-hydroxy- and 4-hydroxyalprazolam analysis in plasma by high-performance liquid chromatography

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Alprazolam is an important triazolobenzodiazepine which has proven efficacy as an anti-anxiety agent. It may also possess antidepressant and anti-panic effects, unusual properties for the benzodiazepine class of drugs [1]. There is a need for newer analytical methods to further study the pharmacology of alprazolam. Published assays for alprazolam have been based on gas chromatography [2] and both normal-phase and reversed-phase high-performance liquid chromatography (HPLC) [3–5]. We have developed an HPLC method which allows quantitation of alprazolam, α-hydroxyalprazolam and 4-hydroxyalprazolam in plasma of rodents or man. The compounds are completely separated from an unidentified peak in occasional plasma samples and can be quantitated at concentrations as low as 2.0 ng/ml. The method is suitable for studying of alprazolam's disposition in single- or multiple-dose pharmacokinetic studies.

EXPERIMENTAL

Apparatus

Chromatography was performed with an automatic HPLC system consisting of an IBM (IBM Instruments, Wellingford, CT, U.S.A.) Model 9533 pump fitted with an IBM 9505 automatic sampler and a 100-μl sample loop. A 25 cm × 4.6 mm IBM reversed-phase column was used containing 5-μm octadecyl (C_{18}) silane as

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the sorbent. An IBM 9523 variable-wavelength UV detector was operated at 214 nm. An IBM CS9000 laboratory computer was used to control the pumps and injector, and to record, store and analyze chromatograms.

Reagents and chemicals

Analytical-grade monobasic and dibasic potassium phosphate and sodium borate were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Ethyl acetate, heptane, methanol and acetonitrile were HPLC grade. Water was passed through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) before use. Alprazolam, α-hydroxyalprazolam, 4-hydroxyalprazolam and triazolam were gifts from UpJohn (Kalamazoo, MI, U.S.A.). Nitrazepam was obtained from Roche Pharmaceuticals (Nutley, NJ, U.S.A.).

Mobile phase and buffers

The mobile phase was a 30:70 (v/v) mixture of acetonitrile and 50 m*M* potassium phosphate (pH 6). A flow-rate of 1.5 ml/min was used at ambient laboratory temperature (22°C).

Standards for calibration graphs

Stock solutions of alprazolam, α-hydroxyalprazolam, 4-hydroxyalprazolam, triazolam (internal standard) and nitrazepam (internal standard) were prepared by dissolving the appropriate amounts of each compound in methanol to make 1 mg/ml free base solutions. Standards for calibration curves were prepared by spiking 1.0-ml aliquots of plasma with diluted stock solutions to make alprazolam and metabolite standards ranging from 2 to 40 ng/ml. The concentration of the two internal standards was 40 ng/ml in each plasma sample. Calibration graphs of the recovered standards were prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system. Graphs were constructed of the peak-height ratio of each compound to internal standard against drug concentration. Two internal standards were used to minimize the possibility of unwanted peaks in samples of patients taking multiple drugs interfering with alprazolam and metabolite quantitation. Triazolam was used to calculate unknown concentrations unless the peak-heigh ratio of the two internal standards suggested a co-eluting peak with triazolam.

Extraction procedures

To 1.0 ml of plasma in a polypropylene tube, 10 μl (40 ng) of internal standard solution, 0.5 ml of saturated solution of sodium borate buffer (pH 9.12) and 4.0 ml of ethyl acetate–heptane (85:15) were added. The tubes were stoppered and placed on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 10 min. The sample tubes were then centrifuged for 10 min at 220 *g*. The ethyl acetate–heptane layer was transferred into a clean polypropylene tube and evaporated to dryness under a gentle stream of nitrogen. The samples were reconstituted with 100 μl of mobile phase and an aliquot was injected onto the HPLC system. Between injections the column was washed with 10 ml of 70% acetonitrile in water to minimize the possibility of late-eluting peaks. Following this wash-out phase,

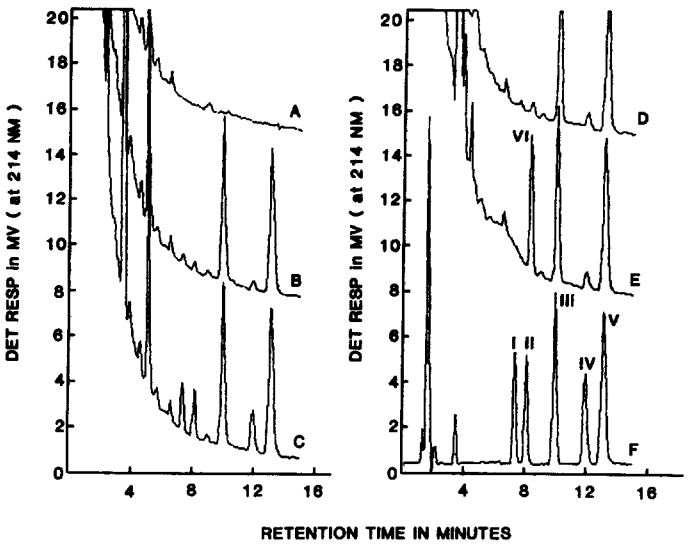

Fig. 1. Chromatograms of (A) extracted blank plasma, (B) a 4 ng/ml extracted calibration plasma standard, (C) a 20 ng/ml extracted calibration plasma standard, (D) extracted plasma from a patient receiving alprazolam therapy collected at 2.5 h post-dose, (E) at 0.25 h post-dose and (F) direct injection of a methanol stock solution containing 40 ng of 4-hydroxyalprazolam (I), 40 ng of α-hydroxyalprazolam (II), 40 ng of nitrazepam (III), 40 ng of alprazolam (IV) and 40 ng of triazolam (V). VI is an unidentified peak which appears in occasional human plasma samples. The tracings in this figure are unretouched computer constructions from raw data stored on disks.

10 ml of mobile phase were pumped through the system to re-equilibrate the column before the next injection.

Recovery and assay validation

Recovery was determined by comparing the peak heights from extracted samples with those obtained from a direct injection of drugs in methanol. Within-day and between-day variability was assessed by extracting spiked samples for reproducibility in the same day or repeatedly over several assay days. A standard curve was prepared daily and the calculated control sample concentrations were compared to the theoretical values for spiked samples.

Application to pharmacokinetic studies

The method was used to quantitate alprazolam plasma concentrations in a single-dose pharmacokinetic study. A 28-year-old healthy male volunteer took 1 mg of Xanax brand of alprazolam (Upjohn) in a fasting state. Blood samples (10 ml) were collected in unstoppered Venoject tubes (Kimble-Turemo, Elkton, MD, U.S.A.) containing sodium heparin as the anticoagulant at various time intervals following the dose by venipuncture of an antecubical vein. In a multiple-dose pharmacokinetic study, blood samples were collected at the end of a steady-state dosage interval from patients receiving Xanax (alprazolam) for therapeutic reasons. In additional studies, Sprague–Dawley rats were given 10 mg/kg intravenous doses of alprazolam and blood collected through an indwelling jugular venous

catheter at various times following the dose. In all cases, blood was centrifuged immediately after collection and plasma stored frozen at −20°C until prepared for assay.

RESULTS

The chromatographic procedure separated alprazolam, its hydroxylated metabolites and internal standards. Retention times for 4-hydroxyalprazolam, α-hydroxyalprazolam, nitrazepam, alprazolam, and triazolam were 7.4, 8.4, 10.5, 12.0 and 13.2 min, respectively. Calibration curves using either internal standard were consistently linear and passed through the origin. Fig. 1 illustrates typical chromatograms of extracted blank plasma, calibration standards, plasma from a patient receiving therapeutic doses of alprazolam and a methanol stock solution.

The recoveries were 96 ± 4.1% (n=8), 93 ± 4.6% (n=8) and 85 ± 6.0% (n=8) for alprazolam, α-hydroxyalprazolam and 4-hydroxyalprazolam, respectively, for 10 ng/ml control samples. Within- and between-day precision studies were run three times (studies A, B and C) over the course of a three-year period. Tables I and II list these precision data obtained by chromatographing aliquots of spiked control plasma.

The results of the single-dose human pharmacokinetic study are shown in Fig. 2. Alprazolam was present in the first sample collected 30 min following the dose and in subsequent samples collected up to 12 h. An elimination half-life, calculated on the basis of apparent logarithmic drug elimination from 3 to 12 h, was 8.0 h. This value is consistent with published literature showing 7–18 h as the range of alprazolam's elimination half-life in healthy subjects [6].

Plasma concentrations of alprazolam from ten psychiatric patients being treated with 1.5–6.0 mg of alprazolam daily for anxiety or mixed anxiety–depression are shown in Table III.

DISCUSSION

Previous HPLC methods have been described for quantifying alprazolam in plasma [2–5]. As their is increasing interest in quantitating active metabolites of psychoactive drugs in pharmacokinetic studies, a major objective of the present study was to develop a method that could also quantitate α- and 4-hydroxyalprazolam. These are the major metabolites of alprazolam, and α-hydroxyalprazolam has been shown to have high affinity for binding to benzodiazepine receptors [7].

The method has been able to consistently quantify 2 ng/ml alprazolam and its metabolites. Our patient samples contained only traces of α-hydroxyalprazolam or 4-hydroxyalprazolam (Fig. 1D and E). Similar findings were recently reported by Smith and Kroboth [8]. However, in plasma from rats analyzed in our laboratory, both these metabolites are easily quantifiable reaching plasma concentrations up to 40% of the concentration of alprazolam in the same samples [9]. Some patient samples collected under steady-state conditions contained an unidentified peak with a retention time slightly longer than that of α-hydroxyalprazolam. This peak can be seen in Fig. 1 as compound VI. In the single-dose healthy-

TABLE I

WITHIN-ASSAY PRECISION DATA FOR ALPRAZOLAM, α-HYDROXYALPRAZOLAM AND 4-HYDROXYALPRAZOLAM FROM SPIKED PLASMA

Compound	Study	*n*	Concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Alprazolam	A	10	10.0 ± 0.70	7.0
	B	10	10.2 ± 0.40	3.9
	C	5	9.5 ± 0.26	2.7
α-Hydroxyalprazolam	A	10	9.9 ± 0.67	6.7
	B	10	10.2 ± 0.86	8.4
	C	5	10.6 ± 0.27	2.5
4-Hydroxyalprazolam	A	10	10.0 ± 0.69	6.9
	B	10	9.8 ± 0.92	9.4
	C	5	10.5 ± 0.51	4.9

TABLE II

BETWEEN-DAY ASSAY PRECISION DATA FOR ALPRAZOLAM, α-HYDROXYALPRAZOLAM AND 4-HYDROXYALPRAZOLAM FROM SPIKED PLASMA

Compound	Study	*n*	Concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Alprazolam	A	10	10.3 ± 0.59	5.7
	B	8	9.8 ± 0.57	5.8
	C	10	11.2 ± 1.15	10.3
α-Hydroxyalprazolam	A	10	9.6 ± 1.28	13.3
	B	8	9.1 ± 0.43	4.7
	C	10	10.4 ± 1.46	14.0
4-Hydroxyalprazolam	A	10	11.8 ± 1.25	10.6
	B	8	8.7 ± 1.65	18.9
	C	10	10.6 ± 0.91	8.6

volunteer study, this peak was absent in plasma obtained immediately before dosing but appeared in the first timed sample after dosing (Fig. 1E). It was absent by 2.5 h (Fig. 1D) after the dose and did not interfere with quantitation of alprazolam.

Alprazolam, α-hydroxyalprazolam and 4-hydroxyalprazolam can undergo ring opening under acid conditions [10]. We used sodium borate in the extraction procedure to adjust the pH to maintain the closed ring structures without causing degradation of the ethyl acetate.

The precision data (Tables I and II) indicate that the assay has acceptable reproducibility. Correlation coefficients for standard calibration curves were con-

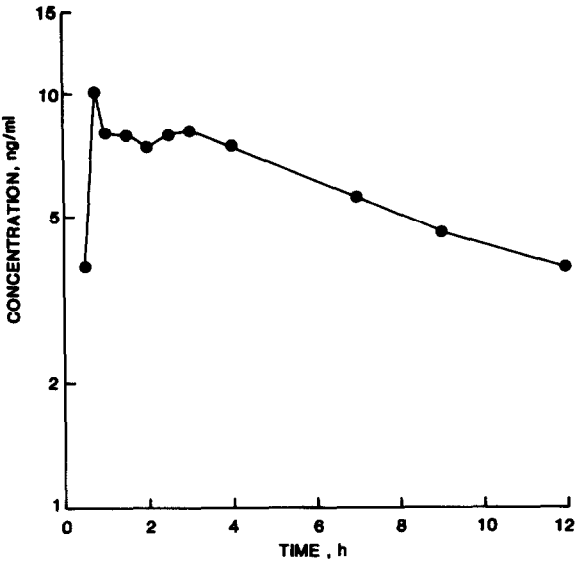

Fig. 2. Alprazolam plasma concentration versus time profile in a healthy volunteer following a single 1-mg oral dose.

TABLE III

ALPRAZOLAM PLASMA CONCENTRATIONS IN PATIENTS TREATED FOR ANXIETY OR DEPRESSION UNDER CLINICAL CONDITIONS

Patient No.	Daily dose (mg)	Alprazolam concentration (ng/ml)
1	1.5	5.2
2	1.5	18.6
3	2.0	9.0
4	2.0	16.2
5	2.5	29.5
6	3.0	21.1
7	4.0	28.3
8	4.0	14.0
9	5.0	34.2
10	6.0	37.9

sistently greater than 0.999. Linearity was demonstrated up to 50 ng/ml (highest concentration tested).

Over a three-year period we have analyzed plasma collected at steady-state conditions from over 100 patients receiving therapeutic doses of alprazolam. Some of these patients were taking other drugs, primarily cyclic antidepressants, and interferences have been rare. Overall, this methodology is sensitive and specific and should prove useful for studying alprazolam disposition in single- or multi-

ple-dose investigations. While alprazolam's major metabolites are absent or are present in only trace amounts in plasma taken from human subjects, this method separates these from alprazolam for quantitation in other species.

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