Quantification of Alprazolam in Serum or Plasma by Liquid Chromatography

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A reversed-phase "high-performance" liquid-chromatographic assay for the quantitation of alprazolam in serum or plasma is described. Serum or plasma is extracted with toluene/isooamyl alcohol (99/1 by vol), evaporated, and reconstituted in the mobile phase. The latter is washed with hexane, then subjected to reversed-phase liquid chromatography and ultraviolet detection at 202 nm. Either U-31485, an alprazolam analog, or lorazepam, a 3-hydroxybenzodiazepine, is satisfactory as internal standards. Major alprazolam metabolites and various other commonly used drugs do not interfere. The useful lower limit of sensitivity for quantification is 2.5 µg/L. Peak height and alprazolam concentration are linearly related from 2.5 to 100 µg/L. For 10 and 20 µg/L concentrations, within-run CVs are 1.4% and 0.9%, and the between-runs CVs 4.8% and 3.2%. Steady-state serum concentrations ranged from 25 to 55 µg/L in patients taking 1.5 to 6.0 mg per day, orally. Preliminary data suggest the method is also suitable for analysis of the structurally similar triazolobenzodiazepine, triazolam.

Additional Keyphrases: chromatography, reversed-phase • antidepressants • triazolam • lorazepam

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-s-triazolo[4,3a][1,4]benzodiazepine), a relatively new triazolobenzodiazepine compound, is anxiolytic in humans (1), and may be effective in the treatment of depression (2-4) and panic disorder (5). Hepatic biotransformation produces hydroxylated alprazolam derivatives and benzophenones, which are rapidly excreted in the urine (6). Of the known metabolites, only a-hydroxyalprazolam has substantial biologic activity, as measured by benzodiazepine-receptor-binding assays or in vivo animal studies (7). Alprazolam metabolites are not present in notable concentrations in human plasma (6,8,9), thus measurement of the parent compound is useful for clinical and toxicological studies.

Alprazolam has been quantified in plasma by electron-capture gas chromatography (9). Pharmacokinetic data have been obtained by gas chromatography (9,10) and by use of [14C]alprazolam (6). A quantitative assay for another triazolobenzodiazepine—the ultra-short-acting hypnotic, triazolam—was reported by Adams et al. (11), who extracted serum with toluene before reversed-phase "high-performance" liquid chromatography (HPLC). This method lacked a sample clean-up step, which we find results in a complicated chromatographic background. We describe here a relatively simple reversed-phase HPLC method for measuring alprazolam in serum or plasma. Our preliminary data suggest that the method is equally suitable for quantification of triazolam.

Materials and Methods

Apparatus

We used a Series 2 HPLC equipped with a variable wavelength detector Model LC-75 and a Model 7120 injector with a 100-µL loop (Perkin-Elmer, Norwalk, CT 06852). Samples were chromatographed on a 250 mm × 4 mm (i.d.) Bio-Sil ODS-10 column (Bio-Rad Laboratories, Richmond, CA 94804).

Reagents

Hexane, acetonitrile, and toluene (all spectrophotometric grade) were purchased from Burdick and Jackson Labs., Muskegon, MI 49442. Isoamyl alcohol, reagent grade, was obtained from J. T. Baker Co., Phillipsburg, NJ 08865, and redistilled in glass.

Stock standard solutions of alprazolam, triazolam, U-31485, lorazepam, and the hydroxyalprazolam metabolites were prepared in methanol to give final concentrations of 1 g/L. Standards stored in the dark at -15 °C have been stable for 10 months.

Working standard solutions were prepared by diluting the stock standards 100-fold with distilled water. A 100 µg/L plasma standard was prepared by adding 50 µL of the aqueous working standard solution to 5 mL of drug-free plasma. Plasma standards ranging from 2.5 to 100 µg/L were prepared in round-bottom 15-mL polytetrafluoroethylene-lined screw-top glass tubes by appropriate dilution of the 100 µg/L plasma standard into drug-free plasma to a final volume of 2 mL.

A working solution of internal standard (1 mg/L) was prepared by dissolving 10 µL of U-31485 stock standard solution into 10 mL of distilled water.

Chromatographic Conditions

The mobile phase consisted of 300 mL of acetonitrile per liter of 50 mmol/L phosphate buffer pH 4.5 (6.9 g of KH₂PO₄ in 1000 mL of water, adjusted to pH 4.5 with c-phosphoric acid). Because 1,4-benzodiazepines undergo a reversible ring-opening reaction under aqueous acidic conditions (12), the mobile phase pH should be 4.5 or greater. Chromatography was performed at 45 °C, with a mobile-phase flow rate of 2.5 mL/min. The column effluent was monitored at 202 nm with the detector set at 0.01 absorbance unit full-scale.

Patients

Plasma or serum samples were obtained from six patients undergoing long-term therapy with oral doses of alprazolam ranging from 1.5 to 6.0 mg/day. "Trough" blood samples were obtained in the morning, just before the patient's next dose.

Procedures

Standard procedure: Add 2 mL of serum or plasma (unknowns, standards, or controls), 100 µL (100 ng) of working internal standard (U-31485), and 0.5 mL of distilled water to the 15-mL screw-top glass tubes and vortex-mix briefly. Extract each sample for 10 min on a rotator.

1 CLIN. CHEM. 30/10, 1652-1655 (1984)
with 10 mL of toluene solvent containing 10 mL of isoamyl alcohol per liter. Centrifuge for 5 min, then transfer the upper (organic) layer to a 15-mL conical test tube and evaporate in a 37 °C water bath under a stream of nitrogen gas. Resuspend the residue in 150 μL of the mobile phase and vortex-mix for 2 min. Add 0.5 mL of hexane, again vortex-mix briefly, and centrifuge for 5 min. Aspirate and discard most of the upper (hexane) layer, inject about 100 μL of the lower phase into the chromatograph.

Interference studies: Potential interference by several common benzodiazepines, tricyclic antidepressants, and other drugs was studied by assaying drug-free plasma supplemented with each compound to a concentration typically encountered in clinical samples. Possible interference by the two major alprazolam metabolites, α-hydroxy- and 4-hydroxyalprazolam, was investigated by assaying drug-free plasma supplemented to 25 μg/L with each of these hydroxy metabolites.

Analytical variables: We calculated analytical recovery at 10, 50, and 100 μg/L by comparing peak heights obtained from supplemented plasma samples with those obtained by chromatographing unextracted standards dissolved in mobile phase. Results for drug concentration in serum ranging from 0 to 100 μg/L were subjected to linear-regression analysis. We determined within-run and between-run precision for two pools of drug-free plasma supplemented with alprazolam to 10 and 20 μg/L.

Assay versatility: We also evaluated the method for another triazolobenzodiazepine, triazolam. Lorazepam, a 3-hydroxy-1,4-benzodiazepine, was also evaluated as an alternative to U-31485 as an internal standard.

Results and Discussion

Figure 1 shows chromatograms obtained from drug-free plasma, drug-free plasma supplemented with alprazolam and internal standard, and plasma from a patient taking 2 mg of alprazolam per day. Retention times for alprazolam and the internal standard, U-31485, were 8.4 and 6.9 min, respectively. We observed no interfering peaks in several samples of drug-free plasma.

Recovery and linearity: Absolute recovery (uncorrected by the standard curve) from plasma samples supplemented with alprazolam to 10, 50, and 100 μg/L was respectively 92%, 94%, and 97% (means of five experiments; CVs were less than 1% for each concentration tested). The peak-height ratios for alprazolam:internal standard were linearly related to the concentrations of the alprazolam standard from 2 μg/L to at least 100 μg/L. The correlation coefficients for each standard curve constructed invariably exceeded 0.99. The slopes of 15 curves, prepared over a period of several months, had a CV of 6% (average regression equation: y = 0.986x − 0.011). The lower limit of detection was approximately 1 μg/L, with a lower limit of quantification of approximately 2.5 μg/L.

Reproducibility: Within-run CVs were 1.4% at 10 μg/L and 0.9% at 20 μg/L (n = 8 each). Between-day CVs were 4.8% and 3.2% for 10 μg/L (n = 12) and 20 μg/L (n = 12), respectively, over a period of four months.

Interferences: We studied 22 compounds for possible interference, including several drugs that might be administered to anxious or depressed patients (Table 1). Imipramine and nortriptyline produced peaks that overlapped with that for alprazolam, while the peaks for desipramine and protriptyline overlapped with the peak for the internal standard (U-31485). Two consecutive washes of the toluene/isoamyl alcohol extract from serum with 0.5 mL of HCl (1.5 mol/L) during sample preparation removed most of the tricyclic antidepressants without affecting alprazolam recovery.

Some interference persisted as a result of the relatively high concentrations of the tricycles as compared with that of alprazolam. The acid wash can also provide evidence that the overlapping peak was a tricyclic or other more strongly basic drug. Concurrent administration of alprazolam and a tricyclic antidepressant would be uncommon.

Triazolam, a triazolobenzodiazepine that cannot be separated from alprazolam by the described method, would not, in clinical practice, be administered with alprazolam.

Patient studies: In six patients taking daily doses of alprazolam ranging from 1.5 to 6.0 mg, steady-state serum
concentrations of the drug were 25 to 55 μg/L (Table 2).

Metabolites: Noninterfering peaks corresponding to the alprazolam metabolites α-hydroxy- and 4-hydroxyalprazolam were observed in chromatograms obtained from drug-free plasma supplemented with these metabolites (Figure 2). For optimal separation of the metabolites, we reduced the volume proportion of acetonitrile in the mobile phase to 26% (Figure 2). With the modified mobile phase, the proposed method should be suitable for analysis of the monohydroxy metabolites (uncorrected recovery 65%; lower limit of detection 1.5 μg/L). Nevertheless, we did not detect monohydroxylated metabolites in a patient with a steady-state alprazolam concentration of 26 μg/L (patient F, Table 2). Moreover, serum from a patient with an alprazolam concentration exceeding 300 μg/L (acute overdose) demonstrated only trace (less than 2.5 μg/L) amounts of these metabolites. Previous studies have not detected alprazolam metabolites in significant concentrations in serum after 1- or 2-mg oral doses in healthy volunteers (6, 9, 10).

Table 2. Illustrative Steady-State Concentrations of Alprazolam in Serum during Long-Term Therapy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Oral alprazolam dose, mg/day</th>
<th>Serum alprazolam, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.5</td>
<td>31.4</td>
</tr>
<tr>
<td>B</td>
<td>2.0</td>
<td>27.0</td>
</tr>
<tr>
<td>C</td>
<td>5.0</td>
<td>41.0</td>
</tr>
<tr>
<td>D</td>
<td>6.0</td>
<td>27.0</td>
</tr>
<tr>
<td>E</td>
<td>6.0</td>
<td>53.1</td>
</tr>
<tr>
<td>F</td>
<td>6.0</td>
<td>26.4</td>
</tr>
</tbody>
</table>

We also assayed plasma samples supplemented with triazolam, another triazolobenzodiazepine compound used clinically as a rapid-acting hypnotic (13). By the described procedure, the triazolam standards demonstrated a retention time of 9.3 min, a linear range from 1 μg/L through at least 100 μg/L, and an uncorrected absolute recovery of 86%. Although we did not evaluate it in depth, the proposed method for alprazolam appears to be suitable for analysis of triazolam.

The requirement for a special internal standard such as U-31485 might limit the implementation of this procedure in clinical laboratories. However, lorazepam, a more readily available 3-hydroxy-1,4-benzodiazepine, can be substituted for U-31485 as an internal standard without changing the overall assay performance. Under the chromatographic conditions described, lorazepam has a retention time of 5.2 min and an uncorrected recovery of 92%. Analysis of a patient's sample with each internal standard yielded the same results both times.

In aqueous acidic conditions, alprazolam is in equilibrium with its corresponding benzophenone compound via a reversible ring-opening reaction (12). Using repetitive-scanning ultraviolet spectroscopy, we detected no formation of benzophenone in solutions of mobile phase at pH 4.5 containing alprazolam, nor were any peaks corresponding to the benzophenone seen by HPLC. However, at lower pH, the equilibrium favors formation of the benzophenone. Although the equilibrium is the same in standards and samples, the mobile phase pH should be 4.5 or greater for maximal sensitivity.

Steady-state serum alprazolam concentrations of 20 to 30 μg/L in patients taking daily doses of 2 to 5 mg were reported by Greenblatt et al. (8, 10). We found concentrations of 25 to 55 μg/L in six patients taking daily doses of 1.5 to 6.0 mg. A clear clinical role for the therapeutic monitoring of other anxiolytic benzodiazepines has not emerged (13). Considering the lack of accumulation of active metabolites, and the recently emerging distinctive clinical features of alprazolam, the measurement of serum concentrations should be helpful in clinical studies.

The study was supported by NIMH grant 1PS0MH30929. We thank Dr. James Collins, Upjohn Co., Kalamazoo, MI 49001 for generous gifts of alprazolam, hydroxyalprazolam metabolites, triazolam, and U-31485. Dr. Dennis Charney provided the patients' blood samples. Lorazepam was a gift of Wyeth Laboratories, Philadelphia, PA 19104.

References