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Note

Gas chromatographic analysis of alprazolam in plasma: replicability, stability and specificity

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Alprazolam is a triazolobenzodiazepine currently in clinical use or under investigation for the treatment of anxiety, depression, and panic disorders [1,2]. Specific and sensitive quantitation of plasma concentrations of alprazolam is necessary for purposes of therapeutic monitoring during clinical investigation or treatment, as well as for studies of alprazolam pharmacokinetics. We previously described an electron-capture gas chromatographic (GC) method for quantitation of plasma alprazolam concentrations [3] which was subsequently modified to allow automatic sampling [4]. This report presents data available on the replicability, stability, and specificity of this assay, based on use since 1981 for clinical and research purposes.

EXPERIMENTAL

The analytical instrument is a Hewlett-Packard Model 5830A or 5840A gas chromatograph equipped with a 15-mCi ^{63}Ni electron-capture detector, automatic sampler, and data processor–integrator. The column is coiled glass, 1.2 m × 2 mm I.D., packed with 3% SP-2250 on 80–100 mesh Supelcoport (clinical packing 1-1767; Supelco, Bellefonte, PA, U.S.A.). The carrier gas is argon–methane (95:5), at a flow-rate of 50 ml/min. Operating temperatures are: injection port and detector, 310°C; column oven, 275°C.

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Solutions of alprazolam and the triazolobenzodiazepine internal standard U-31485 (kindly provided by the Upjohn Company, Kalamazoo, MI, U.S.A.) are prepared by dissolving 10 mg of either compound in 100 ml of methanol. The stock solutions are stored frozen. Working solutions are prepared by diluting the stock solutions 1:100 with methanol or toluene. These solutions are stable indefinitely when stored refrigerated or frozen.

For monitoring of plasma alprazolam concentrations in the range usually encountered at steady state during administration of therapeutic doses [5], 50 ng of the internal standard (50 μl) are added to a series of 13-ml round-bottom culture tubes Calibration tubes are prepared by adding varying amounts of alprazolam (5, 10, 25, 50, 75, 100, 150, and 200 ng). The solvents are evaporated to dryness at 40–50°C under conditions of mild vacuum Drug-free control plasma (0.5–1 ml) is added to the calibration tubes. "Unknown" plasma (0.5–1 ml) is added to all other tubes, containing only internal standard. A 2-ml volume of the extraction solvent (benzene–isoamyl alcohol, 98.5:1.5) is added, and the samples are agitated in the upright position on a vortex mixer for approximately 60 s. After centrifugation for 10 min at 400 *g*, the organic layer is transferred to 2-ml Wheaton automatic sampling vials. The organic solvent is evaporated to dryness, and the residue is redissolved in 225 μl of toluene (containing 15% isoamyl alcohol and 3% of a 1 mg/ml solution of purified soy phosphatides in benzene). A 6-μl aliquot is injected onto the chromatograph using the automatic sampler.

For single-dose pharmacokinetic studies, U-31485 (10 ng) is used as the internal standard, and alprazolam calibration standards are prepared by adding 1, 2.5, 5, 7.5, 10, 15, 20, 25 and 30 ng of alprazolam to calibration tubes. Drug-free control plasma (0.5–1 0 ml) is added to calibration tubes, and 1.0 ml of unknown plasma is added to all other tubes containing only internal standard. The extraction and analysis then proceed as described above [6].

Within-day replicability

Steady state. Plasma samples were obtained from patients receiving usual therapeutic doses of alprazolam during clinical trials. For a series of 193 samples (plasma concentration range: 3–187 ng/ml) analyzed in duplicate on the same day, the coefficient of variation (C.V.) between each pair of duplicate determinations was calculated as the standard deviation divided by the mean of the two determinations, expressed in percent.

Single dose. A similar analysis was performed on a series of 161 samples obtained from subjects who had received single 1-mg oral doses of alprazolam. Plasma concentrations did not exceed 14 ng/ml in all samples.

Between-day replicability

Steady state. "Internal" quality control samples were prepared from a large pool of drug-free control plasma to which was added alprazolam to produce a final concentration of 37.5 ng/ml. The pooled plasma was divided into individual

1-ml aliquots, which were frozen and stored at −5°C. One of these samples was thawed and analyzed along with each daily set of "unknown" plasma samples. Also analyzed with each set of unknowns was an "external" quality control sample, containing 75 ng/ml alprazolam, freshly prepared on the day of analysis.

Single dose. Two "internal" quality control samples (containing 4.7 and 22.9 ng/ml alprazolam), prepared as described above, were thawed and analyzed along with unknown samples from single-dose studies.

Stability of samples

A series of 64 plasma samples from patients receiving usual therapeutic doses of alprazolam during clinical trials (concentrations range: 4–272 ng/ml) were analyzed, refrozen, and stored at −5°C for approximately one year. The samples were then thawed, reanalyzed, and the results from the two determinations done one year apart were compared

Comparison of GC and gas chromatographic–mass spectrometric (GC–MS) assays.

Plasma samples ($n = 212$) obtained from patients receiving alprazolam during clinical trials were divided into two aliquots. Alprazolam concentrations in one of the aliquots was determined by GC with electron-capture detection as described above. In the other aliquot, alprazolam concentrations were determined by GC–MS [7]. The two laboratories were geographically remote, and analysts at neither site had knowledge of the concentrations determined at the other site.

RESULTS AND DISCUSSION

Within-day replicability

For the 193 samples representing steady-state plasma alprazolam levels, the mean C.V. between replicates was 7.3%. In 73% of plasma, the C.V. between replicates was less than 10%. For the 161 samples obtained during single-dose kinetic studies, the mean C V. between replicates was 9.3%. In 70% of samples, the C.V. between replicates was less than 10% (Fig. 1).

Between-day replicability

Across 31 analytic runs (to determine steady-state plasma concentrations) done on different days, the mean measured concentration in the internal quality control sample was 35.3 ng/ml, with a standard deviation (S.D.) of 2.1 and C.V. of 5.9%. For the external quality control sample, the mean value was 76.3 ng/ml, the S.D. was 3.4, and the C.V. 4.5%.

Across 22 analytic runs to determine plasma concentration after single doses, the mean measured concentration in the "low" quality controle sample was 5.2 ng/ml (S.D. = 0.30; C.V. = 5.7%); in the "high" sample, the mean value was 22.8 ng/ml (S.D. = 1 52, C.V. = 6.7%) (Fig. 2).

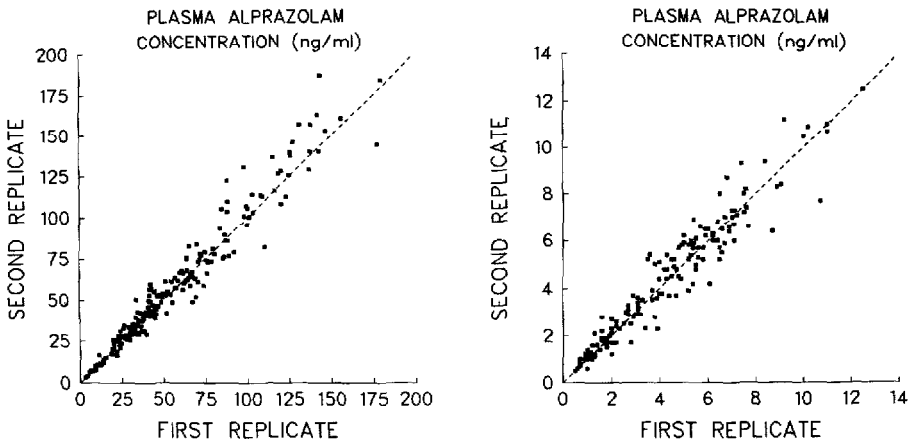

Fig. 1. Left. results of duplicate determinations of plasma alprazolam concentrations in 193 samples from patients receiving usual therapeutic doses of alprazolam during clinical trials The dashed line is the line of identity ($y = x$). Right· results of duplicate determinations in 161 samples from subjects who had received single 1.0-mg oral doses of alprazolam and had plasma concentrations less than 14 ng/ml

Stability

The mean C.V. between results from the two analyses done one year apart was 7.0%, and the C.V. was less than 10% in 84% of the samples (Fig. 3).

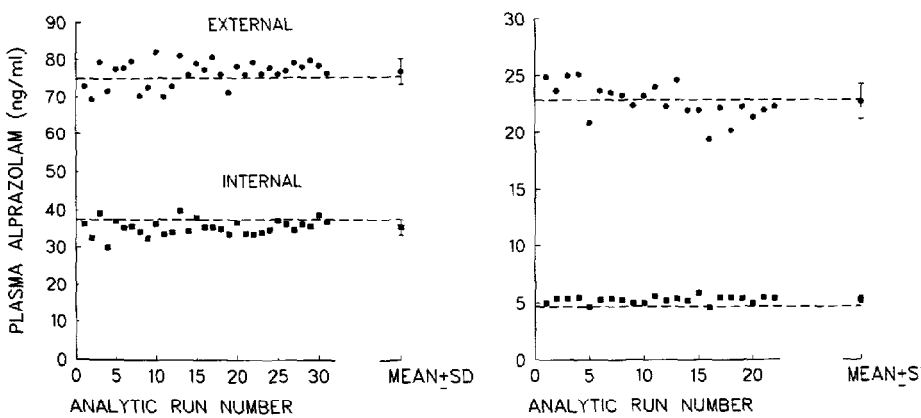

Fig 2. Left: plasma alprazolam concentrations in "external" and "internal" quality control samples (prepared as described in the text) determined during 31 separate analytic runs done on different days. Also shown is the overall mean (± S D) of the 31 individual values. Horizontal dashed lines are the actual target concentrations (75 and 37 5 ng/ml, respectively) Right: plasma alprazolam concentrations in "high" and "low" quality control samples determined during 22 separate analytic runs done on different days, in the context of single-dose pharmacokinetic studies Also shown is the overall mean (± S.D) of the 22 individual values. Horizontal dashed lines are the actual target concentrations (22.9 and 4 7 ng/ml, respectively)

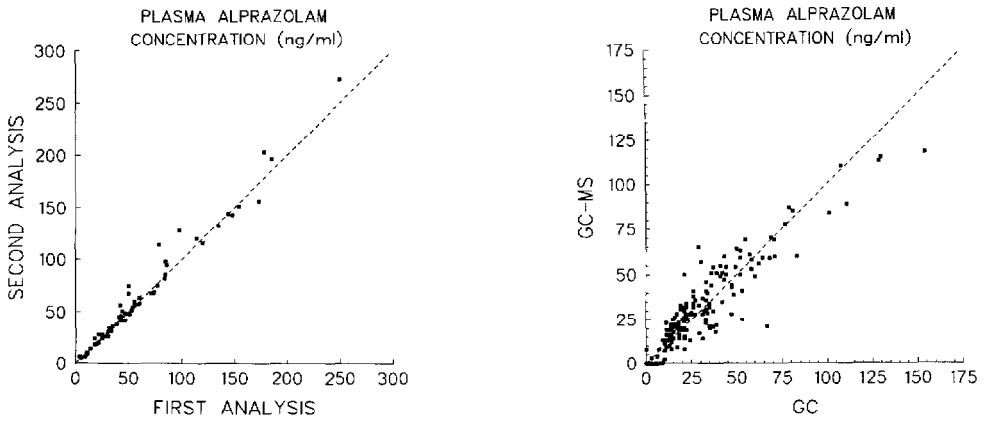

Fig 3 Results of alprazolam plasma concentrations initially determined in a series of 64 samples (x-axis) Samples were stored at −5°C for one year, thawed, and reanalyzed (y-axis). The dashed line is the line of identity ($y = x$).

Fig. 4. Plasma alprazolam concentrations in split aliquots of plasma samples determined in two separate laboratories by GC (x-axis) and by GC–MS (y-axis) The dashed line is the line of identity ($y = x$)

Comparison of GC and GC–MS assays

Alprazolam plasma concentration values obtained by the two methods were highly intercorrelated (Fig. 4). The overall correlation coefficient was 0.93 ($p < 0.0001$) and the slope of the regression line passing through the origin was 0.95. In 77 samples in which the GC method yielded plasma alprazolam values of less than 10 ng/ml, the GC–MS value likewise was also less than 10 ng/ml in 71 samples (92%). In samples having plasma alprazolam concentrations greater than or equal to 10 ng/ml by both methods, the overall mean C V. was 18%, and in 66% of the pairs the C.V. was less than 20%.

Comment

GC with electron-capture detection has now been used for quantitation of plasma alprazolam concentrations in many thousands of samples Evaluation of quality control data indicates good assay performance characteristics in terms of within-day and between-day replicability. Stability of frozen samples for periods of at least one year has also been demonstrated.

Biotransformation of alprazolam in humans reportedly occurs by oxidative metabolism, leading to two or possibly more hydroxylated metabolites [8,9] that are largely excreted in conjugated form [10]. Steady-state concentrations of unconjugated hydroxylated metabolites of alprazolam during chronic therapy with alprazolam are considerably lower than those of the parent compound [5,11], suggesting that clinical activity of alprazolam is largely attributable to the effects of alprazolam itself. Although detectable GC peaks are not produced by hydroxy

metabolites of alprazolam tested to date, the specificity of the GC method cannot be unequivocally validated without comparison to GC–MS. The present comparison of GC and GC–MS methods indicates that values obtained from the two methods are comparable, and that the GC method appears to be specific for intact alprazolam.

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