Simultaneous Gas-Chromatographic Analysis for Diazepam and Its Major Metabolite, Desmethyldiazepam, With Use of Double Internal Standardization

David J. Greenblatt

Electron-capture gas–liquid chromatography was used for simultaneous quantitation of plasma diazepam (I) and its major metabolite, desmethyldiazepam (II). Because concentrations of I can greatly exceed those of II after single doses of I, two benzodiazepine-analog internal standards were added to all samples, thereby allowing reliable quantitation of high concentrations of I and low concentrations of II. After extraction at neutral pH with benzene (containing isooamy alcohol), the organic extract is evaporated and the residue is reconstituted in a small volume of solvent and chromatographed on a 3 % OV-17 column. The sensitivity limits are 2 to 3 ng of I or II per milliliter of original sample, with a CV for identical samples of <5%. The applicability of the method to single-dose pharmacokinetic studies of I in humans is illustrated.

Additional Keyphrases: pharmacokinetics • drug assay

Methods and Materials

Apparatus and Chromatographic Conditions

We used a Hewlett Packard Model 5750 gas chromatograph equipped with a 2-mCi 63Ni electron-capture detector, operated in the pulsed mode with a pulse interval of 150 μs. The column is coiled glass, 182 cm in length by 4 mm i.d., packed with 3% OV-17 on 80/100 mesh Chromosorb WHP (Supelco, Inc., Bellefonte, Pa.). The carrier gas is ultrapure helium (Matheson Gas Products, Gloucester, Mass.), flow rate, 50 ml/min. The purge gas is 95/5 argon/methane (flow rate, 80 ml/min). Operating temperatures are: injection port, 300 °C; column, 280 °C; detector, 320 °C. At the beginning of each working day, the column is primed by injection either of two to three drug-free blank plasma extracts, or of 2 to 3 μl of a 1 g/liter solution of asolectin (Associated Concentrates, Woodside, NY) in benzene. The need for subsequent priming injections depends upon the age of the column, and is assessed by evaluation of the stability of peak-height ratios after repeated injection of the same sample.

Preparation and Analysis of Samples

Ten milligrams of DZ and DMDZ, and of the two internal standard benzodiazepine analogs (I), Ro7-9957 and Ro7-9749 (Figure 1,1 each are dissolved in 2 to 3 ml of absolute ethanol, then diluted to 100 ml with benzene. The stock solutions, stored in amber-colored bottles at 4 °C, are stable for at least a year. Working standards, containing 0.25 to 1.0 mg/liter, are prepared as needed by appropriate dilution with benzene.

A constant amount (usually 100 μl) of a working internal standard mixture, containing Ro7-9957 (0.7 mg/liter) and Ro7-9749 (0.3 mg/liter), is added to a series of conical 40-ml centrifuge tubes equipped with Teflon-lined screw-top caps. The solutions are evaporated to dryness at 40 to 50 °C under mildly reduced pressure. Calibration standards are prepared by adding DZ from the working standard solution to consec-

1 Kindly supplied by Dr. W. E. Scott, Hoffmann-La Roche, Inc., Nutley, N.J.
Fig. 1. Structure of the 1,4-benzodiazepin-2-one nucleus
Structures of specific compounds are:

<table>
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<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
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<td>Diazepam (DZ)</td>
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<td>Cl</td>
<td>H</td>
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<tr>
<td>Desmethyldiazepam (DMDZ)</td>
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<td>Cl</td>
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<tr>
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<td>Ro7-9749</td>
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Fig. 2. A: Chromatogram of a drug-free control plasma extract. B: Chromatogram of the same plasma sample to which was added diazepam (DZ), 100 ng/ml; desmethyldiazepam (DMDZ), 12.5 ng/ml; Ro7-9957, 70 ng/ml; and Ro7-9749, 30 ng/ml.

Fig. 3. Calibration curves showing the linear relation between plasma concentration of DZ or DMDZ and the peak height ratio of the compound to its respective internal standard (Ro7-9957 or Ro7-9749).

Fig. 4. Plasma concentrations of diazepam and desmethyldiazepam during 98 h after a single 5-mg intravenous dose of diazepam given to a healthy female volunteer. Also shown (solid line) is the pharmacokinetic function for diazepam determined by iterative nonlinear least-squares analysis.

Clinical Pharmacokinetic Study

A healthy 29-year-old woman volunteer participated after giving written informed consent. A single 5-mg dose of diazepam was infused directly into an antecubital vein during 15 s. During the subsequent 98 h multiple venous blood samples from the other arm were collected into heparinized tubes. Plasma was separated and frozen until assay. DZ and DMDZ concentrations in all samples were determined as described above.

Plasma DZ concentrations were subjected to iterative weighted nonlinear least-squares regression analysis, to determine pharmacokinetic characteristics of diazepam distribution, elimination, and clearance (13, 14).

Results

Evaluation of the Method

Absolute retention times of DZ, DMDZ, and the two internal standards (Figure 2) depend upon the age of the column, but relative retention times are independent of column age.

A linear relation exists between concentrations of DZ or DMDZ in the calibration standards and the peak-height ratio of each of the compounds to its respective internal standard (Figure 3). The sensitivity limits are about 2 to 3 ng of DZ or
DMDZ per milliliter of original sample. Coefficients of variation for identical DZ samples (n = 8 at each concentration) were: 2.8% at 25 ng/ml; 4.3% at 50 ng/ml; 2.1% at 100 ng/ml; and 3.1% at 200 ng/ml. For DMDZ, the variation of identical samples at 25 ng/ml (n = 8) was 1.2%. The mean deviation between 267 randomly selected sets of duplicate samples containing DZ concentrations ranging from 4 to 360 µg/liter was 3.7%. Residue analysis indicated that extraction of all four compounds is more than 95% complete.

Pharmacokinetic Study

Disappearance of DZ from plasma was best described by the following function consisting of the sum of three exponential terms:

\[ C = 110.4e^{-0.80t} + 132.3e^{-1.26t} + 51.4e^{-0.0219t} \]

where C represents the plasma DZ concentration (ng/ml or µg/liter) at time t (in hours) after the dose (Figure 4). DZ disappearance was mirrored by appearance of its metabolite, DMDZ.

Based on the coefficients and exponents determined from the fitted function, the size of the dose, and the body weight of the subject (49.1 kg), the following pharmacokinetic variables were determined (13, 14):

- Volume of central compartment: 0.35 liter/kg body weight
- Total apparent volume of distribution: 1.89 liter/kg
- Initial (alpha) distribution half-life: 4.2 min
- Intermediate distribution half-life: 32.9 min
- Elimination (beta) half-life: 31.7 h
- Total clearance: 0.69 ml/min per kilogram

Discussion

Electron-capture gas-liquid chromatography is established as a sensitive and specific approach to quantitation of diazepam and desmethyldiazepam in human blood or plasma (1-12). The selectivity of the electron-capture detector obviates the need for extensive sample clean-up; plasma samples from drug-free individuals are consistently free of contaminants appearing at the retention times of DZ and DMDZ or of the two internal standards. Chromatographic peaks corresponding to all four compounds are symmetric, allowing use of peak height rather than peak area to quantitate detector response (15).

The double internal standardization approach greatly facilitates single-dose pharmacokinetic studies of diazepam in humans. Relatively high concentrations of the parent compound are quantitated by using one reference standard (Ro7-9749) of which relatively large amounts are added to the original sample. Low concentrations of DMDZ, generated by hepatic demethylation of DZ, are quantitated with use of the other reference standard (Ro7-9749), added in smaller amounts. When concentrations of the parent compound also are low, the "low" standard (Ro7-9749) can also be used for measuring DZ.

The applicability of the method to clinical pharmacokinetic investigations is illustrated. Consistent with previous reports (16), disappearance of diazepam from plasma following a single intravenous bolus injection appeared to have three exponential phases. This complex pattern is consistent with a three-compartment open pharmacokinetic model, and indicates that diazepam is distributed to different tissues at different rates. The total apparent volume of distribution was estimated at 1.89 liter/kg, indicating extensive overall tissue uptake. Following attainment of distribution equilibrium, elimination proceeded with a typically long half-life of 31.7 h (16, 17). Total metabolic clearance of diazepam was 0.69 ml/min per kilogram body weight. Diazepam appears to be cleared largely by hepatic demethylation (18), yielding the metabolic product DMDZ, the appearance of which in plasma mirrors the disappearance of the parent compound. Because both compounds have similar pharmacologic activity (18), both must be considered in attempts to determine the clinical relevance of the pharmacokinetic profile of diazepam in humans.

Minor modifications of the described method can be used for simultaneous quantitation of DZ and DMDZ during multiple-dose therapy. Chronic dosing with DZ leads to accumulation of both DZ and DMDZ (16, 19, 20). At "steady-state," plasma concentrations of both compounds generally are similar, and they can be estimated, by use of a single internal standard, preferably Ro7-9749, with appropriate modifications of the calibration and standardization techniques.

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References


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Modified Jendrassik–Grof Method for Bilirubins Adapted to the Abbott Bichromatic Analyzer

Leonard Mori

I describe a semi-micro method for use with a discrete analyzer, the Abbott ABA-100, requiring 75 µl of sample for determination of both the total and direct bilirubins. This method utilizes a serum blank. It is simple and reasonably rapid. The azobilirubin formed by both the total and the direct bilirubins produces a reddish-violet color, which is measured at 550 nm. Values obtained are comparable to those by the automated Jendrassik and Grof procedure adapted by Gambino and Schreiber (total r = 0.999, direct r = 0.999). The method is linear to 300 mg/liter. Day-to-day precision (CV) for the total bilirubin was 8.8% for a 8 mg/liter sample (n = 45) and 4.7% for a 57 mg/liter sample (n = 46). The absorbance of hemoglobin and its derivatives (540 nm) caused a false decrease in the measured bilirubin content, the direct being more affected than the total. This problem was alleviated by the use of caffeine and acid blanks.

Many discrete analyzers are now in use in clinical laboratories. A major advantage of such systems is the small amount of sample and reagent(s) required. Many procedures have been adapted successfully to discrete systems, but there still are difficulties with some of them. An example is the bilirubin methodology; total bilirubins can be successfully measured, but determination of the direct still poses problems. A method has been described for total and direct bilirubins with the ABA-100 (1). Certain difficulties were encountered during the use of this method. Not only did the dependence upon a kit (A-Gent Bilirubin Reagent; Abbott Diagnostics Division, South Pasadena, Calif. 91030) increase reagent costs, but special care was necessary to perform the test. A preliminary investigation of this method indicated that occasionally the value for direct bilirubin tended to be higher than expected when run against our manual Jendrassik and Grof method (2). We abandoned the procedure and devised a modification of the Jendrassik and Grof method in which the azobilirubin formed in the first step of their reaction was measured at 550 nm. The resulting procedure is described below.

Materials and Methods

Instrumentation

A Beckman Acta III spectrophotometer was used to obtain the azobilirubin scans shown below in Figure 1. All tests were performed with the ABA-100 Bichromatic Analyzer (Abbott Diagnostics Division Analytical Systems, South Pasadena, Calif. 91030).

Reagents

Caffeine reagent. Dissolve 50 g of caffeine, 75 g of sodium benzoate, and 125 g of sodium acetate in de-ionized water at 60 °C and dilute to 1.00 liter when cool. This reagent is stable for six months at room temperature.

Sulfanilic acid. Dissolve 10 g of sulfanilic acid in de-ionized water containing 15 ml of concentrated hydrochloric acid and dilute to 1.00 liter. This reagent is stable for at least six months at room temperature.

Sodium nitrite, 0.14 mol/liter. Dissolve 1.0 g of sodium nitrite in 100 ml of de-ionized water. This reagent is stable for a week when stored at 4 °C.

Hydrochloric acid, 25 mmol/liter. Using a volumetric pipette, transfer 25 ml of 1.00 mol/liter hydrochloric acid to a 1.00-liter volumetric flask that has been rinsed and half filled with de-ionized water. Dilute to the mark.

Sodium chloride. Add 9.0 g of sodium chloride to a 1.00-liter volumetric flask that has been rinsed and half filled with de-ionized water. Mix until dissolved and dilute to the mark.

Diazo reagent. Mix 10 ml of sulfanilic acid reagent with 0.25 ml of sodium nitrite solution. Prepare just before use.

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