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Note

Lorazepam analysis using liquid chromatography: improved sensitivity for single-dose pharmacokinetic studies

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Analytical methodology for lorazepam in body fluids has been hampered by the extremely high sensitivity required for detection of the low plasma lorazepam concentrations achieved after usual therapeutic dose as well as by thermal instability of the 3-hydroxybenzodiazepine ring, making gas chromatographic methods difficult to standardize. High-performance liquid chromatographic (HPLC) methods are available for determination of benzodiazepine concentrations in human plasma [1–5], but none are sensitive enough to measure the low plasma lorazepam concentrations attained during single-dose pharmacokinetic studies. Gas–liquid chromatography using electron-capture detection (GLC—ECD) has been used with greater sensitivity; however, this methodology requires the use of another 3-hydroxybenzodiazepine as internal standard and the assumption that thermal molecular rearrangement on-column occurs to the same extent with lorazepam and internal standard [6]. Here we present a liquid chromatographic method, comparable in sensitivity to the GLC—ECD method, for analysis of lorazepam after single therapeutic doses. Application of this method to the pharmacokinetic study of lorazepam in a healthy male volunteer is presented. The method is then compared to the GLC—ECD method [6].

EXPERIMENTAL

Apparatus and chromatographic conditions

The HPLC system consisted of a Waters Assoc. Model 510 (Milford, MA, U.S.A.) dual-piston solvent delivery system, a Waters Model 440 ultraviolet

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spectrophotometer operated at 254 nm, and a Waters Model U6K sample injection loop. Detector output was quantitated on a Fisher Model 5000 (Fairlawn, NJ, U.S.A.) chart recorder. The separation system was a 30 cm × 3.9 mm I.D. stainless-steel (10 μm) C_{18} μBondapak reversed-phase column. A methanol–water (50:50) solution was left flowing through the column when not in analytical operation. The mobile phase used for the chromatographic separation was 0.01 *M* sodium acetate–methanol–acetonitrile (47.5:40:12.5) at pH 4.6 run at a flow-rate of 2.2 ml/min. All analyses were done at room temperature.

Materials

Pure reference standards of lorazepam and diazepam were supplied by Wyeth Labs. (Radnor, PA, U.S.A.) and Hoffman-LaRoche Labs. (Nutley, NJ, U.S.A.), respectively. All reagents, analytical grade or better, were purchased and used without further purification, except isoamyl alcohol which was glass-distilled prior to use. The mobile phase was filtered using a 0.22-μm pore size filter, then degassed under mild vacuum.

Stock solutions

Standard solutions were prepared by dissolving 10 mg of diazepam (internal standard) in 100 ml of methanol and 10 mg of lorazepam in 100 ml of methanol. Sequential dilutions to 1 μg/ml were made. Potassium phosphate buffer (1 *M*) was made by dissolving 136.1 g of KH_2PO_4 in 1 l of distilled water, and adjusting to pH 7 with 1 *M* K_2HPO_4 solution.

Preparation of samples

A 100-μl volume of stock solution (1 μg/ml) containing 100 ng diazepam was added to a series of screw-capped (Polytef-lined) 15-ml glass tissue culture tubes. A 1-ml sample of unknown plasma was added to each tube. Calibration standards were prepared by adding 2.5, 5, 10, 25, 50, and 100 ng lorazepam to consecutive tubes. Drug-free plasma was added to each calibration standard and was analyzed with the calibration standards and each set of unknown samples.

Extraction procedure

Phosphate buffer (2 ml) was added to each tube. The plasma and buffer were vortexed for 30 s. An 8-ml volume of hexane–isoamyl alcohol (95:5) was added to each tube. This was shaken gently by hand for 10 min, followed by 1 min of vortexing. It was then centrifuged at 4°C for 10 min at 400 *g*. The organic layer was transferred to a clean 15-ml glass tube and the aqueous layer discarded. A 2-ml volume of 6 *M* hydrochloric acid was then added. The tubes were shaken for 10 min, vortexed for 1 min, and centrifuged at 400 *g* for 10 min. The organic layer was discarded. Sodium hydroxide (6 *M*) was slowly added (approximately 2 ml) to achieve a pH greater than 7.0, and 2 ml of phosphate buffer were added. This was vortexed for 10 s. To the aqueous basic extraction mixture, 8 ml of hexane–isoamyl alcohol (95:5) were added. This was shaken for 10 min, vortexed for 1 min, and then centrifuged for 10 min at 400 *g*. The organic layer was aspirated into glass conical tubes and evaporated to dryness at 40°C with nitrogen. The residue was rinsed from the sides with hexane–iso-

amyl alcohol (95:5) and again dried with nitrogen. The residue was then dissolved in 40 μl of methanol and 20–40 μl of the extraction solution were then injected into the chromatographic system.

Pharmacokinetic study

A healthy 39-year-old male volunteer received lorazepam, 2 mg intravenously, by bolus injection, after giving written informed consent. Multiple blood samples were drawn over the following 48 h, and the plasma was separated for determination of lorazepam concentration.

Data analyses

Plasma lorazepam levels for the pharmacokinetic study were analyzed by iterative non-linear least-squares weighted regression analysis, as described elsewhere [7, 8]. The pharmacokinetic parameters, distribution half-life, total clearance, and total volume distribution were then determined from the derived coefficients and exponents. Correlation analysis was performed by calculating the correlation coefficient of the least-squares regression line through the data points using the equation $y = mx + b$, m being the slope and b the intercept of the line.

RESULTS

Evaluation of the method

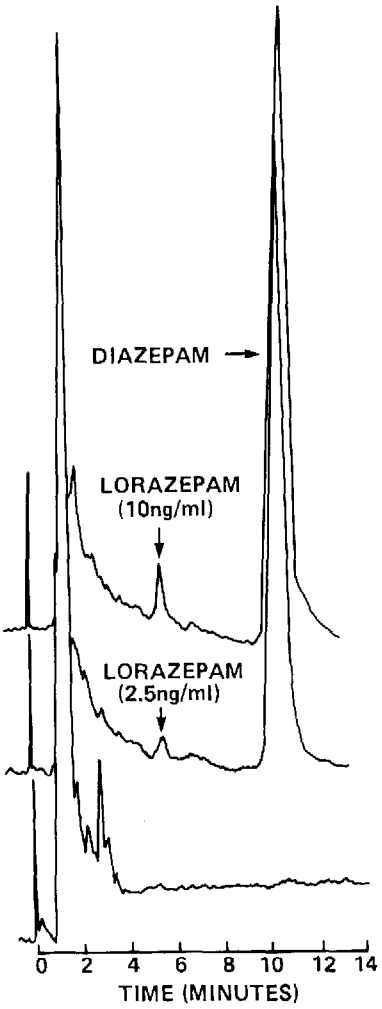
As can be seen from Fig. 1, lorazepam and diazepam give symmetric, well resolved peaks. Drug-free plasma was always free of contaminant peaks at the retention times of the compounds. The peak-height ratio of lorazepam to diazepam was linear to at least 500 ng/ml lorazepam. The correlation coefficient (r) of the generated standard curves was always greater than 0.99.

The sensitivity of the method was 2.5 ng/ml. A typical concentration 32 h after 2 mg lorazepam intravenously was approximately 3 ng/ml; therefore, lorazepam can be reliably measured to that time point after a single dose.

Analysis of twenty standard curves over a six-month period indicated that the day-to-day coefficient of variation of the slopes was 11%. Within-day coefficients of variation for identical samples of lorazepam were: at 2.5 ng/ml, 9.8% (n = 5); 5 ng/ml, 10.0% (n = 5); 10 ng/ml, 4.2% (n = 5); 20 ng/ml, 2.6% (n = 4); 25 ng/ml, 4.6% (n = 5); 50 ng/ml, 4.0% (n = 6).

A series of 50 plasma samples from pharmacokinetic studies was analyzed using both GLC–ECD [6] and HPLC to cross-validate the methodology in determination of lorazepam plasma concentrations (Fig. 2). The correlation coefficient between the methods was 0.95 and the slope of the regression line was 0.96.

Under described conditions, the retention times for lorazepam and diazepam were 5.2 and 9.2 min, respectively. Comparison of these retention times to a series of benzodiazepines was made to demonstrate that other benzodiazepines may be extracted by the same procedure (Table I) and that among drugs evaluated, only oxazepam (another 3-hydroxybenzodiazepine) had chromatographic characteristics similar to those of lorazepam.



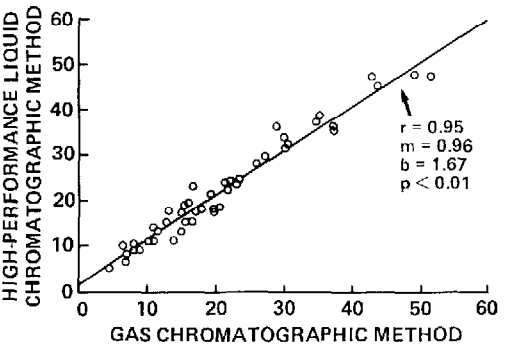


Fig. 1. Liquid chromatograms of extracted plasma (1 ml); with drug-free plasma (lower), 2.5 ng/ml lorazepam from spiked plasma (center), and 10 ng/ml lorazepam from a volunteer sample given 2 mg lorazepam intravenously (upper).

Fig. 2. Comparison of lorazepam determination using HPLC and GLC–ECD methods on 50 plasma samples obtained from pharmacokinetic studies. The line was determined by least-squares regression analysis.

Pharmacokinetic study

Fig. 3 shows lorazepam concentrations and the pharmacokinetic function for the described subject. Derived pharmacokinetic variables are listed in Table II.

DISCUSSION

This paper describes a selective, sensitive method to quantitate lorazepam in plasma by HPLC using ultraviolet detection and describes its application to pharmacokinetic studies. Of greatest value is the ability to monitor levels of

TABLE I

BENZODIAZEPINE RETENTION TIMES UNDER THE CHROMATOGRAPHIC CONDITIONS DESCRIBED IN THE TEXT

Drug	Retention time (min)
Midazolam	7.2
Flunitrazepam	6.0
Clonazepam	5.0
Oxazepam	5.2
Diazepam	9.2
Lorazepam	5.2
Flurazepam	7.2
Temazepam	5.0
Nitrazepam	3.5

TABLE II

DERIVED LORAZEPAM PHARMACOKINETIC PARAMETERS AFTER A SINGLE 2-mg INTRAVENOUS DOSE IN A 30-YEAR-OLD MALE

Distribution half-life (min)	5.4
Elimination half-life (h)	14.6
Volume of distribution at steady state (l)	73.0
Total clearance (ml/min)	58.3

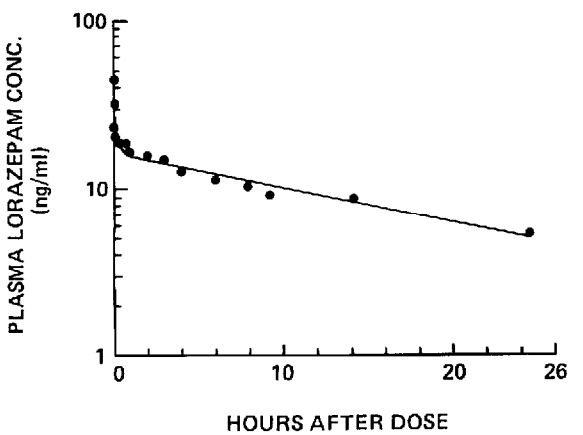


Fig. 3. Plasma lorazepam concentrations and pharmacokinetic function from a single-dose study of a 39-year-old male. See Table II for the pharmacokinetic variables.

lorazepam as low as 2.5 ng/ml, making it a realistic option to the GLC—ECD method. Kabra et al. [4] used a method similar to this, but a different solvent extraction (diethyl ether) and a different mobile phase. However, with the ether extraction there is background interference, and sensitivity is approximately one order to magnitude less (20–30 ng/ml). The HPLC method of Walmsley and Chasseaud [9] used for lorazepam estimation in monkey plasma suffers from the same problem when applied to human plasma. Furthermore, a possible advantage over the GLC—ECD method is that this method is

measuring unchanged lorazepam rather than the product of an on-column molecular rearrangement of lorazepam and, on that basis, may be subject to less variability.

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