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## Note

# Improved method for the determination of diazepam and N-desmethyldiazepam in plasma using capillary gas chromatography and nitrogen–phosphorus detection

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Diazepam (Fig. 1) is a member of the benzodiazepine class of drug compounds. It is widely prescribed for the treatment of several disorders including tension, anxiety, skeletal muscle spasms, sleep disorders, and status epilepticus [1–3]. Diazepam is metabolized in the liver to N-desmethyldiazepam (nordiazepam) (Fig. 1). Since both the parent drug and its primary metabolite are active, a complete understanding of its pharmacological activity should include an analysis of both species [3]. The therapeutic blood levels of diazepam are in the ng/ml range, thus requiring sensitive methods of detection. Gas chromatography (GC) equipped with electron-capture detection [3–5] is the analytical method most widely used to achieve the necessary sensitivity. High-performance liquid chromatographic (HPLC) methods [5–7] are widely usedf, but classically lack sensitivity for low levels.

Microbore HPLC [8,9] has been used to enhance sensitivity, and microbore HPLC with on-line preconcentration has resulted in 4 ng/ml detection limits [8]. Nitrogen–phosphorus detection (NPD) has been used in conjunction with GC for diazepam analysis [10] but analysis of the N-desmethyl metabolite and quantitative capability was not firmly established.

We describe in this paper an analytical method for the determination of both diazepam and nordiazepam in plasma which uses capillary GC–NPD. This method offers several advantages over currently used methods including increased sensitivity, increased dynamic working range, and minimal interferences.

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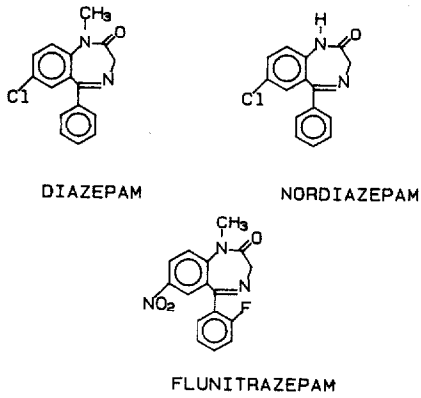


**Fig. 1. Structures of diazepam and its primary metabolite N-desmethyldiazepam (nordiazepam) and the internal standard flunitrazepam.**

## EXPERIMENTAL

The solvents used in this method, methanol, toluene, and methylene chloride, were HPLC grade and purchased from Fisher Scientific (Springfield, NJ, U.S.A.). Diazepam and N-desmethyldiazepam were both provided by Hoffman-La Roche (Nutley, NJ, U.S.A.). Individual stock solutions of each were prepared in methanol at a concentration of 1 mg/ml. Standards containing both analytes were prepared by taking various aliquots of each stock solution and adding to blank plasma. The final standard concentrations of diazepam and N-desmethyldiazepam were 500 and 100, 250 and 50, 150 and 30, 100 and 20, 75 and 15, 50 and 10, 20 and 4, 10 and 2, 5 and 0 and 1 and 0 ng/ml. The two lowest standards contained only diazepam added to blank plasma. Flunitrazepam (Hoffman-La Roche) (Fig. 1) was used as an internal standard at a final concentration of 50 ng in 50 $\mu$l.

The extraction procedure involves adding 50 $\mu$l of internal standard to a 0.5-ml plasma sample in a 100 × 13 mm borosilicate glass test tube with a polypropylene screw-top cap (Fisher Scientific). Methylene chloride (4 ml) was added to the extraction tube which was vigorously shaken on a mechanical shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 20 min at 250 cycles per minute. Following centrifugation at 839 *g* for 8 min, the upper serum layer was removed by suction aspiration and discarded. The lower organic layer was transferred to a 75 × 12 mm borosilicate glass culture tube (American Scientific Products, McGraw Park, IL, U.S.A.) and evaporated to dryness using a vacuum vortex-evaporator (Buchler, Fort Lee, NJ, U.S.A.) heated to 37°C. The dried residue was reconstituted with 200 $\mu$l of toluene, transferred to a 0.3-ml autosampler vial (32 × 6 mm) (Phase Separations, Queensferry, U.K.), and a crimp cap with a chlorobutyl rubber septum was applied. A 5-$\mu$l aliquot was then injected into the gas chromatograph.

A Hewlett-Packard Model 5890 gas chromatograph equipped with a nitrogen–phosphorus detector was used for the analysis along with an HP 7673 autosampler and HP 3392 integrator. The column was an OV-17 methylphenyl silicone bonded-phase fused-silica capillary column (15 m × 0.32 mm I.D., 0.5 mm film

thickness) (QuadRex, New Haven, CT, U.S.A.). Purified helium (Airco, Murray Hill, NJ, U.S.A.) at a flow-rate of 30 ml/min was used as the carrier gas. Detector gas flow-rates were: air, 80 ml/min; hydrogen, 3 ml/min. The oven temperature program for optimal peak separation was as follows: initial oven temperature, 230°C, hold time, 1.5 min; temperature ramp to 300°C at 40°C/min; hold time, 3.0 min; temperature ramp to 325°C at 25°C/min, hold time, 1.0 min. Other settings during operation were: injection port temperature, 250°C; detector temperature, 325°C; splitless injection with a non-silanized glass insert.

## RESULTS AND DISCUSSION

Analysis of blank plasma containing no drug showed no interfering peaks (Fig. 2). The diazepam, N-desmethyldiazepam, and internal standard peaks are well separated and symmetrical (Fig. 3). Chromatograms from subjects receiving diazepam were not significantly different from spiked plasma chromatograms. The capillary column maintained satisfactory resolution and peak shape throughout the injection of approximately 4000 plasma samples. A 50-ml volume of the inlet side of the column was broken off to maintain good chromatography approximately every 500 samples. The second temperature ramp is used as a "burn-off" to elute any strongly retained compounds. Without this second temperature ramp, strongly retained compounds gradually accumulate until saturation occurs. Once binding sites become saturated, the compounds elute at lower temperatures and interfere with analysis.

Unsilanized glassware was used throughout the entire study including the use of an unsilanized glass insert in the injection port. This is because it was observed that using silanized glassware resulted in low recovery of diazepam due to binding to the silanized glassware. This finding was consistent with what other investigators have observed [3]. Unknown plasma samples were analyzed in batches of

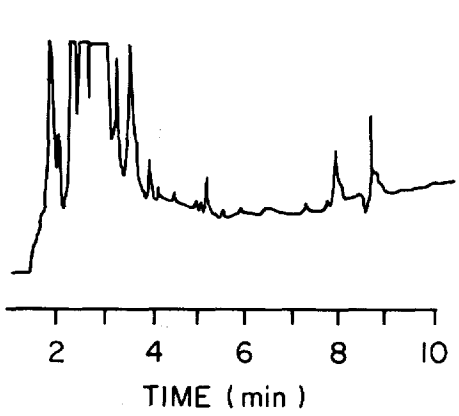


Fig. 2. Example chromatogram of a drug-free plasma extract.

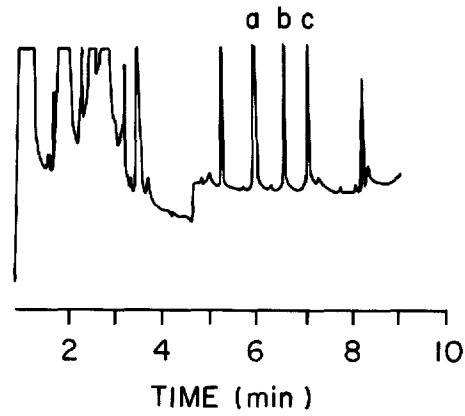


Fig. 3. Example chromatogram of an extracted plasma sampled containing 150 ng/ml diazepam (a), 330 ng/ml nordiazepam (b) and flunitrazepam (c) as the internal standard.

TABLE I

BETWEEN-DAY ACURACY AND PRECISION OVER A FOUR-MONTH PERIOD

| Spiked concentration (ng/ml) | $n$ | Assayed concentration (mean ± S.D.) (ng/ml) | Precision (R.S.D., %) | Accuracy (% error) |
|---|---|---|---|---|
| *Diazepam* | | | | |
| 300 | 105 | 294 ± 17.0 | 6.0 | 2.0 |
| 150 | 102 | 146 ± 7.6 | 5.1 | 2.6 |
| 50 | 101 | 49 ± 3.5 | 7.1 | 2.0 |
| 25 | 88 | 24 ± 2.4 | 10.0 | 4.0 |
| *N-Desmethyldiazepam* | | | | |
| 60 | 105 | 60 ± 4.5 | 7.5 | 0.0 |
| 30 | 102 | 29 ± 2.1 | 7.2 | 3.3 |
| 10 | 101 | 10 ± 1.1 | 11.0 | 0.0 |
| 5 | 88 | 5.1 ± 0.9 | 17.0 | 2.0 |

50–100. Spiked quality control samples were intersperced among unknowns and made up at least 10% of each batch.

Standard curves were prepared from chromatograms of extracted plasma to which known amounts of diazepam and N-desmethyldiazepam were added. Peak-area ratios of each analyte to that of the internal standard were plotted versus concentration. The sensitivity of the method, as defined as twice the level of background noise, was found to be 1 ng/ml for diazepam and 2 ng/ml for N-desmethyldiazepam. These levels were found to be quantifiable at 25.7 and 23.5% relative standard deviation ($n = 10$) for diazepam and N-desmethyldiazepam, respectively. The standard curves were linear up to 500 ng/ml for diazepam and 100 ng/ml for nordiazepam with mean correlation coefficients of 0.9997 and 0.9998, respectively.

The method was evaluated for between-day and within-day accuracy and precision. Accuracy was evaluated as the percentage error of the mean assayed concentration relative to the spiked concentration at four different levels of each analyte and was found to be within 5% throughout the range studied (Table I). The accuracy measured on a within-day basis was poor for N-desmethyldiazepam (Table II) but within expected limits for a 95% confidence interval for a single measurement based on the between-day precision data (Table I). Adequate precision was indicated by the relative standard deviations in Tables I and II. Absolute recovery (extracted versus direct injection) was also determined at four different levels of concentration. Diazepam demonstrated recoveries of 91.0, 92.4, 115.6, and 96.9% for concentrations of 500, 100, 50, and 1 ng/ml, respectively. N-Desmethyldiazepam demonstrated recoveries of 98.1, 89.3, 121.5, and 109.3% for concentrations of 100, 20, 10, and 1 ng/ml, respectively.

## CONCLUSION

In conclusion, an analytical method was developed for the quantitative determination of diazepam and its primary metabolite N-desmethyldiazepam in plasma.

TABLE II

WITHIN-DAY ACCURACY AND PRECISION ($n=12$)

| Spiked concentration (ng/ml) | Assayed concentration (mean ± S.D.) (ng/ml) | Precision (R.S.D., %) | Accuracy (% error) |
|---|---|---|---|
| *Diazepam* | | | |
| 300 | 307.1 ± 12.2 | 4.0 | 2.4 |
| 150 | 142.9 ± 4.5 | 3.1 | 4.7 |
| 50 | 47.9 ± 1.5 | 3.1 | 4.2 |
| 25 | 22.6 ± 1.4 | 6.3 | 9.2 |
| *N-Desmethyldiazepam* | | | |
| 60 | 52.9 ± 1.5 | 2.8 | 11.8 |
| 30 | 25.7 ± 1.0 | 3.9 | 14.3 |
| 10 | 8.2 ± 0.4 | 4.7 | 18.0 |
| 5 | 4.3 ± 0.2 | 4.4 | 14.0 |

The extraction entails a simple, rapid procedure, and the method shows a high degree of specificity and sensitivity towards both analytes with minimal interferences. It offers several advantages over electron-capture methods including a wider linear working range, fewer interferences, comparable sensitivities, use of methylene chloride instead of benzene as the extracting solvent, and avoidance of radiation encountered with the electron-capture detector. Also, the use of derivatizing agents commonly used in electron-capture methods was not necessary with the current method. We have evaluated our method over a one and a half year period and have analyzed over 10 000 samples with a minimum of problems encountered.

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