L.J.M. is the recipient of the Research Career Development Award CA 00112.

References

CLIN. CHEM. 25/7, 1296–1300 (1979)

Analysis for Diazepam and Nordiazepam by Electron-Capture Gas Chromatography and by Liquid Chromatography

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We describe the use of electron-capture gas chromatography or reversed-phase “high-performance” liquid chromatography for concurrent analysis for diazepam and nordiazepam in serum. In the gas-chromatographic analysis we use of a new chemically deactivated stationary liquid phase, SP 2250-DB (Supelco, Inc.), resulted in improved chromatographic sensitivity and peak symmetry for the two benzodiazepines as compared to that obtained with either OV-17 or OV-1 phases. Steady-state concentrations of diazepam and nordiazepam in serum as determined by gas–liquid chromatography correlated closely with those found by liquid–liquid chromatography.

Additional Keyphrases: monitoring therapy • drug assay • anticonvulsants • analyses for benzodiazepines generally

Diazepam is used in treating anxiety, insomnia, psychiatric disturbances, seizures, and minor pain. Since 1970, it has been one of the most widely prescribed drugs, and during the past several years it has been the most prescribed drug (1). Several methods have been proposed for its determination in biological specimens, with gas chromatography involving electron-capture detection being the technique most frequently described (1–9).

There are two major metabolic transformations of diazepam in man: its N-demethylation to nordiazepam (N-desmethyl-diazepam), which is an active metabolite, and hydroxylation of 3-hydroxy-diazepam, which is then demethylated to oxazepam, a metabolite that does not reach significant concentrations in serum during therapy with diazepam. (10, 11). In contrast, nordiazepam can equal or exceed the diazepam concentration in serum after chronic dosing with diazepam, and indeed some investigators believe that this metabolite has the more sedative and mood-depressant effect (12–14). In addition, the large variations reported in steady-state concentrations among patients suggest the need for accurate monitoring of both the drug and its active metabolite (15, 16).

In our laboratory, analyses for nordiazepam by gas-chromatographic methods, with OV-1 and OV-17 stationary liquid phases, were unrewarding owing to the chromatographic properties of the metabolite. The procedures did not provide a suitable chromatographic peak of nordiazepam, particularly in therapeutic concentrations, without special treatment (loading) of the column before each analysis. Use of these two silicone phases constitutes the separative mechanism for practically all previously reported methods, except for the method of Whelpton and Curry (7), who used OV-225.

Recently, some published procedures involve "high-per-
formance” liquid chromatography for separating and detecting diazepam and nordiazepam (17–21). This technique eliminates the problem of poor peak symmetry or “tailing” for nordiazepam, which often is associated with gas-chromatographic methods. Bugge (17) and Perchalski and Wilder (18) used the adsorption mode of separation; others (19–21) used a reversed-phase column.

This note describes two chromatographic procedures for the concurrent analysis for diazepam and nordiazepam without derivatization. The procedure requires only 1 mL of plasma. One procedure is an electron-capture gas–liquid chromatographic technique utilizing a stationary liquid phase that has improved chromatographic properties for nordiazepam; the other is a liquid-chromatographic procedure, with a reversed-phase column.

Materials and Methods

Chromatography

We used a Model 5830A gas chromatograph (Hewlett-Packard, Avondale, PA 19211) equipped with a N2 electron-capture detector and a 0.7 m × 2 mm (i.d.) glass column packed with GP 3% SP2250-DB on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA 16823) for the analyses. Column, injector, and detector temperatures were 235, 300, and 350 °C, respectively. Carrier gas (95% argon–5% methane) flow rate was 60 mL/min.

For liquid-chromatographic analyses we used a Model 5000 high-performance liquid chromatograph (Varian Associates, Palo Alto, CA 94303) equipped with a Micro Pak MCH-10 column, a 10-μL loop valve injector, and a 254-nm ultraviolet detector. Chromatographic conditions were as follows: mobile phase, methanol/water (3/1 by vol); flow rate, 2.0 mL/min; column temperature, 40 °C.

Reagents

Diazepam and nordiazepam. These were obtained from Hoffman-La Roche, Inc., Nutley, NJ 07110. A 1 g/L solution of each drug was prepared, in methanol.

Prazepam. This internal standard was obtained from Warner-Lambert Research Institute, Morris-Plains, NJ 07950. A 1 g/L stock solution was prepared, in methanol. An aliquot of the stock solution was diluted with ethyl acetate to give a concentration of 100 μg/mL (mg/L). An appropriate volume of the latter solution was added to the extraction solvent (hexane/ethyl acetate, 70/30 by vol) to yield a praze- pam concentration of 0.1 μg/mL.

Ethyl acetate and hexane. These were obtained from Fisher Scientific Co., Pittsburgh, PA 15219 (cat. no. E-191 and H-300, respectively). Both were pesticide grade.

Methanol (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

Water. Purified by passage through a 0.5-μm filter and then through carbon and ion-exchange resin columns.

Procedure

Prepare plasma samples utilized in the standard curve by adding the appropriate amounts of diazepam and nordiazepam to 16 × 150 mm screw-cap tubes, evaporating the solvent in a stream of air, adding 10 mL of blood plasma, and vortex-mixing.

For analysis, place 1.0 mL of plasma or serum and a scoop (Coors no. 02 porcelain spoon) of a NaHCO3/Na2CO3 (3/1 by wt) mixture into 16 × 150 mm tubes. Use a plastic funnel, 67 mm in diameter, with the stem cut to a length of 10 mm, to add the salt mixture. After vortex-mixing the contents of the tubes briefly, extract the serum by adding 10.0 mL of hexane/ethyl acetate (70/30 by vol) containing 0.1 μg of prazepam per milliliter to the samples, and shake the mixture for 15 min on an Eberbach shaker. After subsequent centrifuging for about 5 min at 2000 rpm, transfer the organic layer to another 16 × 125 mm screw-cap tube and evaporate the solvent under a stream of dry air at 55 °C. Reconstitute the contents of the tube in 1 mL of ethyl acetate and briefly vortex-mix. Inject 5 μL of the resulting solution into the electron-capture-equipped gas–liquid chromatograph. For analysis by liquid chromatography, reconstitute the residue into 50 μL of methanol and inject 10 μL.

Results

The recently introduced column packing, SP 2250-DB, is sufficiently deactivated to permit its use in analyses for many basic drugs and their metabolites, without derivatization. To evaluate this phase for the analysis of certain benzodiazepine compounds, we injected equal amounts of diazepam, nordiazepam, and prazepam on each of three columns, SP 2250-DB, OV-17, and OV-1. Nordiazepam shows substantially less tailing on the SP 2250-DB phase, chromatographic efficiency is increased, and retention time is shortened (Figure 1). Peak shapes and electron-capture detector sensitivities for both diazepam and prazepam are also improved with the SP 2250-DB liquid phase. Typical chromatograms from the gas-chromatographic analysis of a plasma standard and a patient’s sample on the deactivated phase are presented in
Table 1. Retention Times for Benzodiazepines

<table>
<thead>
<tr>
<th>Drug</th>
<th>Gas chromatography Retention time, min</th>
<th>Liquid chromatography Retention time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>0.97</td>
<td>2.8</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>2.01</td>
<td>2.4</td>
</tr>
<tr>
<td>Prazepam</td>
<td>1.59</td>
<td>3.5</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>2.01</td>
<td>—</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>1.81</td>
<td>1.9</td>
</tr>
<tr>
<td>Medazepam</td>
<td>0.43</td>
<td>—</td>
</tr>
<tr>
<td>Hydroxyflurazepam</td>
<td>2.96</td>
<td>2.0</td>
</tr>
<tr>
<td>Demoxepam</td>
<td>2.13</td>
<td>1.7</td>
</tr>
<tr>
<td>Desalkylflurazepam</td>
<td>1.63</td>
<td>2.0</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>—</td>
<td>1.8</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>0.71</td>
<td>2.0</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>—</td>
<td>1.7</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>1.37</td>
<td>3.1</td>
</tr>
<tr>
<td>Norchlordiazepoxide</td>
<td>1.51</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* Concentrations, 10 μg/mL.  
* Possible interference under column conditions described.

Figure 2. Similar results for the liquid-chromatographic analysis are illustrated in Figure 3.

Figure 4 shows correlation between diazepam and nordiazepam concentrations in serum from 18 patients on continuous therapy for a period long enough to achieve a steady-state drug concentration in their blood. Nordiazepam concentrations averaged 21% higher than that of the parent drug in the patients on extended therapy. The average steady-state diazepam and nordiazepam concentrations were 467 and 568 μg/L, respectively. Diazepam concentrations by the two methods were highly correlated: \( r^2 = 0.997; \) slope = 1.033; intercept = 22.77. A similar comparison of the two methods for nordiazepam yielded a correlation coefficient, slope, and intercept of 0.998, 1.013, and 16.4, respectively (Figure 4).

We used the same extracts for both types of analyses. Before the extraction with hexane/ethyl acetate, the pH of the serum samples was adjusted to pH 9.7 with the described mixture of crystalline NaHCO3 and Na2CO3. Analytical recovery for both diazepam and nordiazepam was quantitative (102.6 ± 2.6% and 100.3 ± 3.8%, respectively) to at least 2000 ng/mL (2 mg/L). The coefficients of variation (CV) for within-day gas-chromatographic analyses of diazepam and nordiazepam were 1.5 and 4.7%, respectively. For within-day liquid-chromatographic analyses, CV’s for diazepam and nordiazepam were 1.7 and 2.4%, respectively. Day-to-day CVs for gas chromatography were 2.4% for diazepam and 3.6% for nordiazepam; for liquid chromatography they were 2.5% and 3.3%, respectively.

We examined 14 different benzodiazepines in concentrations of 10 mg/L, to see if they would interfere with the analysis for diazepam and nordiazepam (Table 1). The different benzodiazepines were as a rule more satisfactorily resolved from diazepam, nordiazepam, and prazepam with gas than with liquid-chromatographic methods. Compounds with similar retention times to those for diazepam/nordiazepam can be resolved from them at lower column temperatures or by use of longer chromatographic columns. Carbamazepine, carbamazepine epoxide, N-desethyl methaximide, hydroxyphenylhydantoin, phenytoin, methsuximide, ethosuximide, primidone, PEMA, phenobarbital, and caffeine had significantly shorter retention times (1.5–1.8 min.) than the benzodiazepines on the reversed-phase column and were not detected by the gas-chromatographic system. Various tricyclic antidepressants—doxepin, desmethyl doxepin, imipramine,
Desipramine, protriptyline, amitriptyline, and nortriptyline—did not interfere with either method.

**Discussion**

With use of the new phase, SP 2250-DB, there was a considerable reduction in gas-chromatographic tailing of nordiazepam and improved peak symmetry for diazepam and prazepam. The more symmetrical peaks improve quantitation by peak area and increase the sensitivity for analyzing subtherapeutic concentrations, particularly in specimens of small volume.

The gas-liquid chromatographic procedure compared favorably to a liquid-chromatographic method that provided well-resolved, symmetrical peaks for diazepam, nordiazepam, and prazepam. The reversed-phase columns used in the liquid-chromatographic technique are becoming the column of choice, because a wide variety of drugs can be separated and the columns require relatively little maintenance and preparation.

The extraction procedure presented is relatively rapid, and is suitable for both types of analyses for diazepam and nordiazepam in serum in therapeutic concentrations. The salting-out technique provides for excellent analytical recovery of the two drugs and simultaneously establishes an optimal pH for their extraction. The hexane/ethyl acetate extractant provides for efficient removal of the solvent after extraction because it is the top layer.

The limit of sensitivity for both methods is 50 ng for a 1-mL sample. The gas-chromatographic procedure provides a time interval of less than 3 min, considerably shorter than that for other similar methods, which require 6–12 min. Preliminary studies indicate that each of the methods can be adapted to the analysis of many additional benzodiazepine compounds.

The technical assistance of Rebecca Olivares, John Sulak, and Esther Sanchez is deeply appreciated. This research was supported in part by grant R01 MH 26431-02 from the National Institute of Mental Health, NIH, Bethesda, MD.

**References**


Micro-Scale Method for Liquid-Chromatographic Determination of Chloramphenicol in Serum

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We describe the use of “high-performance” liquid chromatography to measure chloramphenicol in as little as 25 μL of serum. Serum is treated to precipitate proteins with acetonitrile containing p-nitroacetanilide as an internal standard. Chloramphenicol is eluted with a mobile phase of methanol in pH 7.0 phosphate buffer (35/65 by vol). The drug is measured at 278 nm and simultaneously monitored at 254 nm; interfering substances are detected by examining the 278 nm/254 nm absorbance ratios. This method is sensitive to less than 0.5 mg/L and the standard curve is linear to at least 50 mg/L. Inter-day precision ranged between 3–6%. We encountered no interference from endogenous compounds or from other drugs we tested.

Additional Keyphrases: monitoring therapy • antibiotics • drug assay

Chloramphenicol is a potent antibiotic with broad-spectrum antimicrobial activity (1). Its effectiveness in treating Hemophilus influenzae meningitis and other diseases caused by bacteria resistant to β-lactam antibiotics has led to its increased use (2). Despite its therapeutic importance, it must be used cautiously because of potentially lethal side effects: aplastic anemia and the “gray-baby syndrome” as well as reversible bone-marrow suppression (1, 3). The latter two effects are directly related to the serum concentration of chloramphenicol. Because of its relatively low therapeutic index and poor correlation between dose and concentration in serum, measurement of serum chloramphenicol during therapy is essential. Most susceptible Gram-negative bacteria are inhibited by serum concentrations between 5 and 15 mg/L (4). As the concentration in serum increases above the therapeutic range, the frequency and severity of toxic side effects increase progressively.

Numerous methods have been developed for measuring chloramphenicol in serum: colorimetric (5), microbiological (6), and gas-chromatographic (7). The routine clinical utility of each of these methods is limited by one or more of the following: lack of sensitivity or specificity, lengthy or complex analysis, poor precision, or requirement for a large serum sample. Recent modifications of a radioenzymatic assay for chloramphenicol (8) have obviated these problems, but this assay still may not be practical for many clinical laboratories because of the lack of a commercial source of the acetyltransferase enzyme and the need for liquid scintillation counting equipment.

We present here a reversed-phase “high-performance” chromatographic method that is simple, rapid, accurate, precise, and for which less than 25 μL of serum is required. Sample preparation consists of precipitating proteins with acetonitrile containing the internal standard p-nitroacetanilide. Each analysis requires less than 15 min. The method is sensitive and will detect chloramphenicol in concentrations as low as 0.5 mg/L. Our method uses the absorbance ratio technique (9, 10) to check for potential interference by monitoring simultaneously the absorbance at 278 nm and 254 nm.

Recently several groups have published methods for determination of chloramphenicol by high-performance liquid chromatography (11–13). The procedure of Nilsen-Ehle et al. (11) does not use an internal standard for quantitation, an omission that could result in poor precision if introduced into the routine clinical chemistry laboratory; the two other procedures (12, 13) require a more lengthy sample preparation involving organic-solvent extraction. In addition, in none of these procedures are absorbance ratios used to check for interfering substances.

Materials and Methods

Apparatus

We used a Model 204 high-performance liquid chromato-