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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF DIAZEPAM AND NORDIAZEPAM IN PLASMA*

VIDMANTAS A. RAISYS*, PATRICK N. FRIEL, PATRICIA R. GRAAFF, KENT E. OPHEIM and ALAN J. WILENSKY

Department of Laboratory Medicine and The Epilepsy Center, University of Washington, Harborview Medical Center, Seattle, WA 98104 (U.S.A.)

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SUMMARY

A one-step method for extraction of diazepam, nordiazepam, and internal standard into toluene is followed by chromatographic separation and detection with either dual-wavelength high-performance liquid chromatography or electron-capture gas—liquid chromatography. Agreement between the two methods was excellent for diazepam ($r = 0.99$, $n = 38$) and good for nordiazepam ($r = 0.96$, $n = 79$) over a concentration range that included subtherapeutic, therapeutic, and toxic plasma levels.

INTRODUCTION

The benzodiazepines are widely used as antianxiety, antispasmodic, and antiepileptic drugs [1]. Patients treated chronically with diazepam exhibit significant plasma concentrations of the active metabolite nordiazepam, as well as the parent drug. The pharmacological actions of two newer benzodiazepines, clorazepate and prazepam, are a result of their biotransformation to nordiazepam [2, 3]. Thus techniques which measure plasma levels of diazepam and nordiazepam are sufficient for plasma level monitoring in patients receiving diazepam, clorazepate, or prazepam. The minimum therapeutic level for diazepam in treatment of acute anxiety is reported to be 0.4 μg/ml [4]; the minimum therapeutic level for diazepam or nordiazepam in epileptic patients is 0.5 μg/ml [5, 6].

In this communication we report a simplified extraction procedure for diazepam, nordiazepam, and internal standard which yields extracts suitable for

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analysis by high-performance liquid chromatography (HPLC) with dual-wavelength monitoring, or by gas–liquid chromatography (GLC) with electron-capture (EC) detection. The two methods of chromatographic separation and detection are compared, and a case study with monitoring of diazepam levels, after intravenous injection of diazepam for control of status epilepticus, is presented.

EXPERIMENTAL

Reagents

Diazepam, nordiazepam, prazepam and nitrazepam were obtained from Hoffmann-LaRoche (Nutley, NJ, U.S.A.). Stock solutions of each drug were prepared in methanol or ethanol (1 mg/ml). Working standard solutions were prepared by appropriate dilutions of stock solutions.

Acetonitrile, glass distilled and of ultraviolet grade was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other reagents used were analytical grade and solvents were nanograde.

HPLC mobile phase was prepared by adding 360 ml acetonitrile to 640 ml of 10 mmol/l phosphate buffer pH 6.0. The solution was filtered through a 0.22-μm filter (type GS, Millipore, Bedford, MA, U.S.A.).

Instrumentation

A Hewlett-Packard Model 5713 gas chromatograph equipped with 15 mCi ^{63}Ni electron-capture detector was used. A 1.8 m × 2 mm I.D. glass column pretreated with 5% dimethyldichlorosilane in toluene packed with 3% OV-17 on Chromosorb W HP 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.) was found most suitable. The column was conditioned by heating at 325°C for 4 h without carrier gas flow, then at 265°C for 12 h with full carrier gas flow. The following gas chromatographic parameters were used: carrier gas methane—argon (5:95), flow-rate 30 ml/min; column temperature 265°C isothermal; inlet temperature 250°C; detector temperature 350°C and attenuation was set at 512. The EC detector was removed from the gas chromatograph at least once a month and cleaned by rinsing with 200 ml absolute methanol.

A Waters Model 204 high-performance liquid chromatograph was used. The flow cell of the Model 440 detector (254 nm) in this system was connected in series to Model 450 variable-wavelength detector (230 nm). A reversed-phase column μBondapak C_{18} (10 μm particle size) 30 cm × 4 mm I.D. was maintained at room temperature. A Waters guard column (3 cm) filled with Bondapak C_{18} Corasil (37—50 μm particle size) was connected to the inlet end of the column (all of the preceding components were obtained from Waters Assoc., Milford, MA, U.S.A.). The absorbance detectors were connected to a dual-pen recorder (Omniscribe, B-5000; Houston Instruments, Austin, TX, U.S.A.).

Procedure

Plasma (1.0 ml), internal standard (0.75 μg nitrazepam for HPLC, 1.00 μg prazepam for EC—GLC), 0.5 ml borate buffer pH 9.0, and toluene (5 ml for HPLC 3 ml for EC—GLC), were combined in glass-stoppered extraction tubes. The drugs were extracted by mechanical shaking of the tubes for 5 min. Following centrifugation, as much of the toluene phase as possible was transferred

to a clean tube and evaporated to dryness. In the EC—GLC procedure, the residue was resuspended in 100 μl of acetone—hexane (20:80, v/v) and 5 μl were injected into the chromatograph. In the HPLC procedure, the residue was dissolved in 50 μl of methanol, and 5 μl were injected into the liquid chromatograph with a mobile phase flow-rate of 2.4 ml/min. The column effluent was monitored at 230 nm (0.01 a.u.f.s.) and 254 nm (0.005 a.u.f.s.).

RESULTS AND DISCUSSION

Chromatograms from our methods are presented in Figs. 1 and 2. Fig. 1A shows a serum blank carried through the procedure and analyzed by HPLC. The chromatogram in Fig. 1B is serum supplemented with diazepam, nordiazepam and nitrazepam (internal standard). Similar chromatograms are obtained by EC—GLC as is shown in Fig. 2.

The concentration of diazepam and nordiazepam in both methods was determined from standard curves using the peak height ratio method (height of the drug/height of internal standard, vs. drug concentration). In the HPLC method

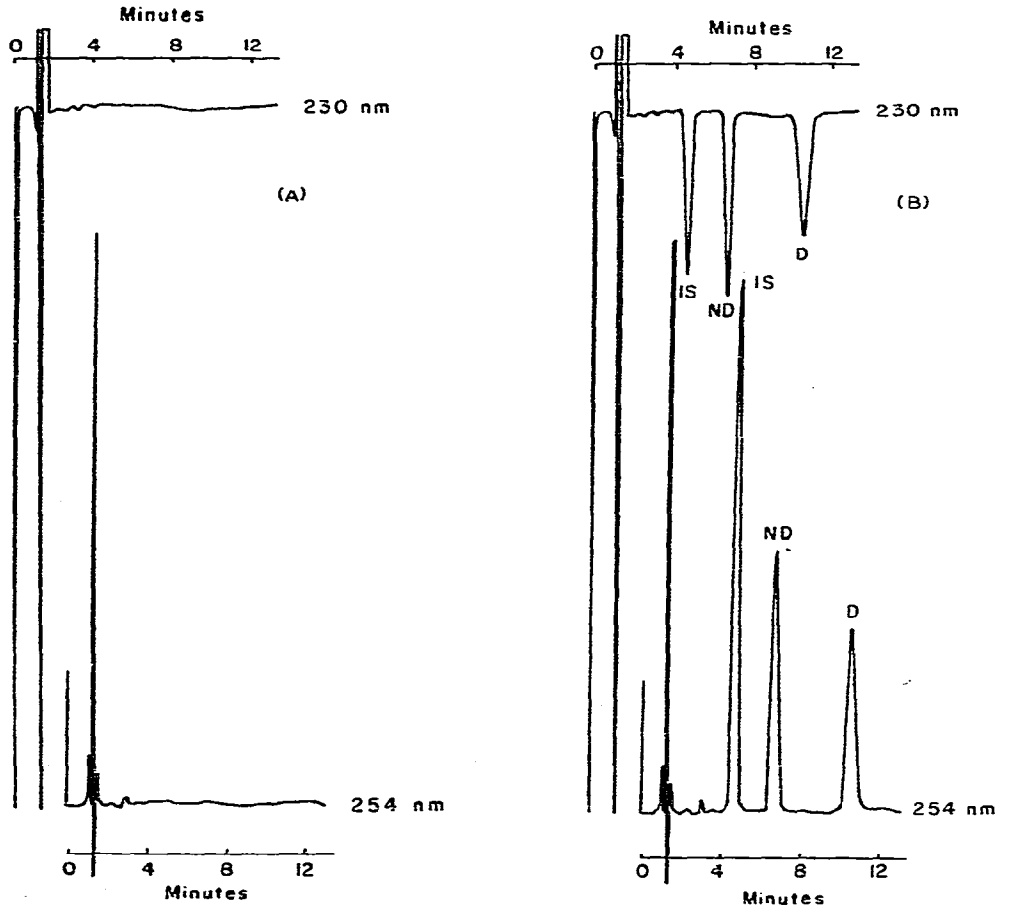

Fig. 1. HPLC chromatograms of plasma blank (A) and plasma extract (B) containing nitrazepam (IS), nordiazepam (ND), and diazepam (D).

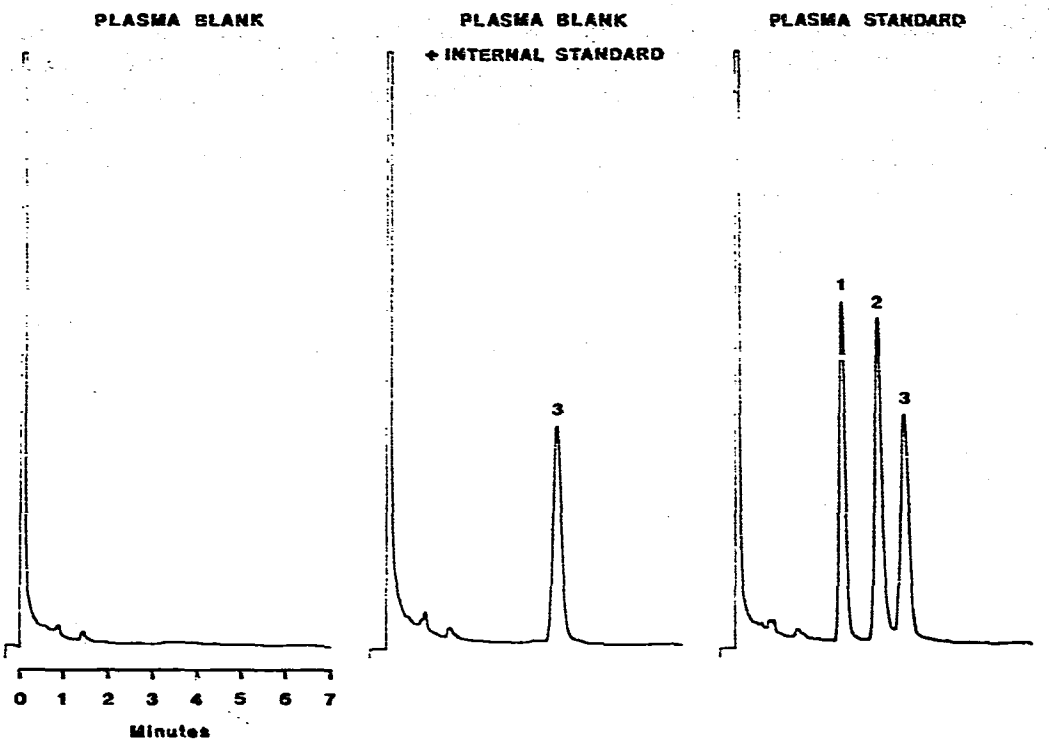

Fig. 2. GLC chromatograms of plasma blank, blank plus internal standard (prazepam) and standard containing diazepam (1), nordiazepam (2), and prazepam (3). Note that there is no apparent decomposition of prazepam to nordiazepam on column.

absorbance at 254 nm was used for quantitation. The peak height ratios are linearly related to drug concentration up to at least 5 μg/ml for each method.

After correction for aliquot loss, the recovery of each of the benzodiazepines studied was greater than 90% with the one-step toluene extraction in both methods.

The precision of the methods was evaluated both within-run and between-runs and was found to be acceptable. The within-run coefficient of variation (C.V.) by the HPLC method was 1.6% for both diazepam and nordiazepam with a mean concentration 2.5 μg/ml (n = 10). At a concentration of 0.7 μg/ml the C.V. was 1.5% and 7.1% for nordiazepam and diazepam respectively (n = 6). The C.V. by GLC was 1.4% for nordiazepam ($\bar{X}$ = 0.5 μg/ml) and 0.4% for diazepam ($\bar{X}$ = 0.5 μg/ml, n = 6). The between-run precision by HPLC was 4.3% for both nordiazepam and diazepam (n = 15) at a mean concentration of 0.7 μg/ml. At the concentration of 2.5 μg/ml the C.V. was 2.6% for nordiazepam and 3.6% for diazepam (n = 21). The between-run precision by GLC was 6.5% for diazepam at a concentration of 0.37 μg/ml and C.V. of 5.7% for nordiazepam at a concentration of 0.76 μg/ml (n = 16).

Correlation studies between the two methods using patient specimens (n = 38, diazepam and n = 79, nordiazepam) showed good agreement. The linear regression line was $y = 0.006 + 1.07\,X$ for nordiazepam with correlation coefficient r = 0.96 and standard error of estimate of 0.240 (Fig. 3). The linear

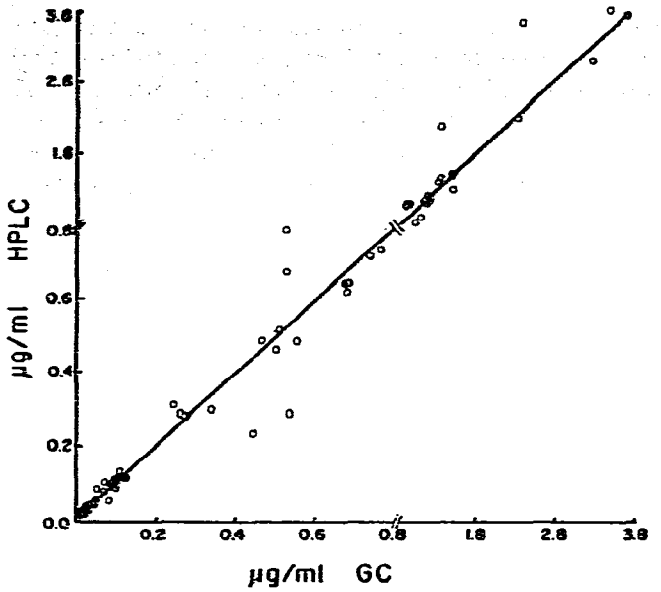

Fig. 3. Comparison of GLC and HPLC results for 79 nordiazepam samples.

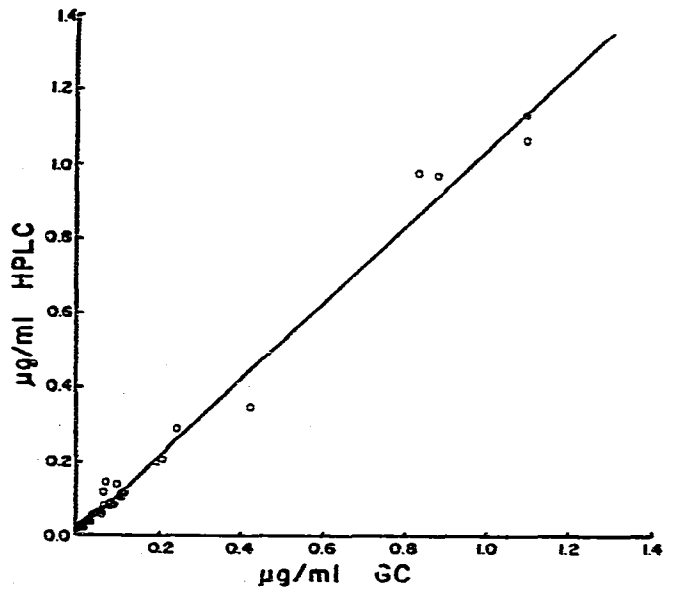

Fig. 4. Comparison of GLC and HPLC results for 38 diazepam samples.

regression line for diazepam was $y = 0.010 + 1.03\ X$ with correlation coefficient of 0.99 and standard error of estimate of 0.036 (Fig. 4).

In the HPLC method we monitored the column effluent at two different wavelengths, 230 nm and 254 nm. The peak height ratio (230/254) was calculated for each drug and internal standard. The ratio is characteristic for each drug and is independent of concentration. Deviation from the established ratio even though the peaks are symmetrical indicates an interfering substance is

being co-eluted. Such samples should be reanalyzed by another method or the mobile phase should be modified to separate the interfering substance. The mean and standard deviation of ratios for diazepam, nordiazepam and internal standard are shown in Table I. There was some variation in the ratio from day to day and the ratio should be established daily from the standards. When the ratio for the drugs or internal standard deviates by 5% or more an interference from another substance is suspected.

TABLE I

ABSORBANCE RATIO MONITORING

n	Drug	Peak height ratio (230 nm/254 nm) ± S.D.
7	diazepam	1.67 ± 0.01
7	nordiazepam	1.74 ± 0.01
9	internal standard	0.84 ± 0.02

In addition to diazepam and nordiazepam, serum chlordiazepoxide concentrations can be determined using the proposed HPLC method. The precision of chlordiazepoxide determination was evaluated and the between-run C.V. was found to be 5.8% at a mean concentration of 0.77 μg/ml ($n = 15$).

None of the commonly prescribed anticonvulsant drugs interfered in the two methods. We have not encountered any interfering substances in the HPLC method by monitoring the two-wavelength ratio. The only drug interference observed in the EC—GLC method was caused by chlorpromazine, which has a retention time of 0.935 relative to diazepam, and is not fully resolved from diazepam in chromatograms where both compounds are present [7]. Use of prazepam as the internal standard in the EC—GLC procedure for determining nordiazepam levels in patients receiving prazepam might lead to an erroneous elevation in the internal standard peak height, and incorrectly low nordiazepam concentrations. However, no prazepam was detected in plasma of subjects receiving single doses of the compound, indicating that the biotransformation to nordiazepam is very rapid [3]. Several other unmarketed benzodiazepines are available from their manufacturers as alternative internal standards [8, 9].

Fig. 5 shows the serum concentration—time curve for a patient who received 5 mg diazepam intravenously for treatment of absence status epilepticus. Diazepam levels were determined by EC—GLC and verified by HPLC. After the injection the patient's seizures stopped and the electroencephalogram normalized for approximately 30 min, when behavioral and electroencephalographic seizures recurred at a diazepam level of 0.116 μg/ml (0.149 μg/ml nordiazepam was also present at this time, primarily due to administration of chlorazepate on the previous day). The dramatic effectiveness and brevity of action of diazepam in status epilepticus are substantiated by its serum level profile after intravenous injection [10].

Many benzodiazepine procedures have used benzene, or a combination of benzene and a more polar solvent, as extraction solvents [8, 9, 11—13]. We have substituted toluene for benzene in this procedure because it is equally efficient in extracting the compounds of interest, and poses a lower risk of chronic poisoning to technologists who are regularly exposed to its vapors [14].

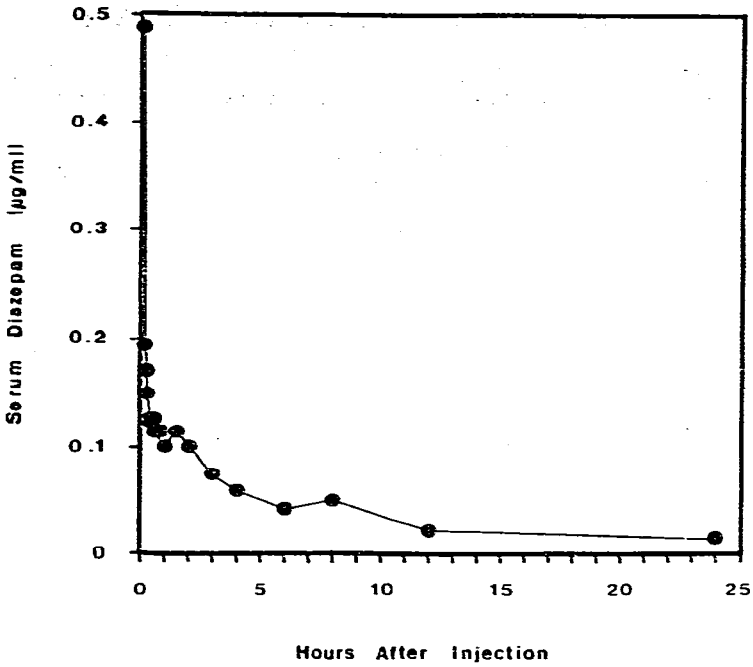

Fig. 5. Serum concentration—time profile for diazepam (5 mg intravenously in an epileptic patient (35 years old, male).

Toluene was used alone, since addition of more polar solvents did not improve the extraction yield of benzodiazepines, but resulted in more rapid contamination of the EC detector (presumably because the extraction residue contained more plasma lipids), which necessitated more frequent detector cleaning and maintenance.

The EC—GLC method described in this paper has been in routine use for two years, and has been adapted, with minor modifications, for the determination of clonazepam in serum [15]. The HPLC method has been in routine use for one year and continuing comparisons of HPLC and EC—GLC results have demonstrated reliable agreement between the two methods, over a range of concentrations that include subtherapeutic, therapeutic, and toxic plasma levels.

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