Journal of Chromatography, 416 (1987) 212–218
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3556

Note

Microsample determination of diazepam and its three metabolites in serum by reversed-phase high-performance liquid chromatography

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(First received September 8th, 1986; revised manuscript received December 19th, 1986)

Diazepam and chlordiazepoxide are the two most widely prescribed antianxiety drugs [1]. Many of their metabolites are also pharmacologically active; among them, N-desmethyldiazepam has been studied most extensively and shown to produce physical dependence in animals [2]. Consequently, it is essential that an assay for these drugs be capable of separating and quantifying the metabolites as well as the parent compounds. Methods have been developed using gas chromatography (GC) with electron-capture detection (ECD) to monitor the plasma level of diazepam and its metabolites [3–10]. Although the electron-capture detector has the sensitivity to determine N-desmethyldiazepam and diazepam, it lacks the sensitivity for oxazepam and temazepam, two other active metabolites. By using a benzene extraction procedure and an SP-2250 column, Löscher [9] reported quantification of diazepam and its three metabolites rapidly and sensitively with GC–ECD. The disadvantage of this method is the health risk associated with repeated exposure to benzene. Furthermore, oxazepam, demoxepam and chlordiazepoxide are not thermally stable and therefore not suited for GC analysis.

Most high-performance liquid chromatographic (HPLC) assays of benzodiazepines [11–20] require plasma sizes of 0.2–1 ml and thus are more suitable for clinical evaluation where the sample volume is less restrictive. However, in research with small laboratory animals, sample size is limited. This is particularly the case in pharmacokinetic studies where it is advantageous to trace temporal changes in blood levels of the drug and its metabolites in the same animal where small sample size is vital for maintaining the hemodynamics of the animal.

We report a simple HPLC method capable of quantitative analysis of diazepam

0378-4347/87/$03.50

and chlordiazepoxide and their metabolites in small samples. By using a single extraction procedure and the commercially available 2 mm I.D. column, the present method can analyze sample sizes as small as 50 μl. An added advantage of using the 2 mm I.D. column is a reduction in solvent consumption by up to 80%, compared to the 4.6 mm I.D. column, without any changes in equipment.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a dual-piston Model 510 pump (Waters Assoc., Milford, MA, U.S.A.), a Model 70-10 sample injection valve, a Model 70-11 loop filler port equipped with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.), a Model 163 variable-wavelength detector (Beckman Instruments, San Ramon, CA, U.S.A.) operated at 228 nm and an LCI-100 integrator (Perkin-Elmer, Norwalk, CT, U.S.A.). The separation was performed on an Ultrasphere C_{18} column (5 μm particle size, 150×2 mm I.D., Altex, San Ramon, CA, U.S.A.). A pre-column filter (2 μm, Waters Assoc.) was also used.

Reagents and standards

HPLC-grade acetonitrile, methanol and diethyl ether were purchased from Fisher Scientific (Springfield, NJ, U.S.A.). All the other chemicals were reagent grade. The 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0) was prepared as described by De Silva et al. [6].

Diazepam, N-desmethyldiazepam, temazepam, chlordiazepoxide hydrochloride, demoxepam and desmethylchlordiazepoxide were gifts from Hoffmann-LaRoche (Nutley, NJ, U.S.A.). Oxazepam was a gift from Wyeth Labs. (Philadelphia, PA, U.S.A.). Working standards of each drug were prepared by appropriate dilutions of the stock solutions. Drugs in methanolic solution remain stable for at least seven days.

HPLC mobile phase was prepared by adding methanol and acetonitrile to a 0.056 *M* sodium acetate buffer which had been adjusted to pH 4.0 with glacial acetic acid (50:5:45, v/v/v). The flow-rate was set at 0.3 ml/min and normally operated at a pressure of 138 bar (2000 p.s.i.).

Sample preparation

For calibration standards, 25 μl of the internal standard (1 μg/ml demoxepam) and 50 μl of the drug standard were pipetted into a 15-ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen. A 50-μl blank serum sample, 100 μl of the 1 *M* borate buffer (pH 9.0) and 2.5 ml of diethyl ether were then added to the sample, vortex-mixed for 30 s, followed by centrifuging at room temperature for 5 min at 1100 *g*. The ether layer was carefully transferred to a 5-ml conical centrifuge tube and evaporated in an evaporator (Pierce, Rockford, IL, U.S.A.) at 40°C under nitrogen. The residue was resuspended in 50 μl of the mobile phase and then 100 μl of *n*-hexane were added in order to remove the colloidal lipids [20]. The mixture was vortex-mixed for 2 s and the hexane layer was removed immediately with a disposable pipette. If necessary, the remaining

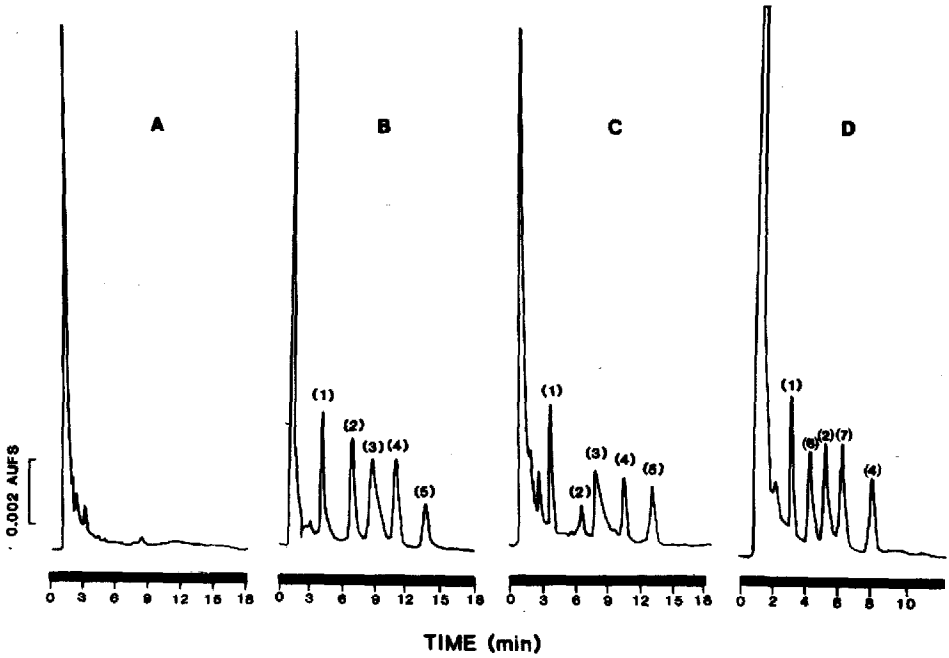

Fig. 1. Chromatograms of (A) rat serum blank, (B) rat serum containing 0.25 μg/ml oxazepam, N-desmethyldiazepam and diazepam and 0.5 μg/ml temazepam taken through the extraction procedure, (C) a 50-μl rat serum sample obtained 15 min after 8 mg/kg diazepam intraperitoneal injection, (D) rat serum containing 0.25 μg/ml demoxepam, desmethylchlordiazepoxide, oxazepam, chlordiazepoxide and N-desmethyldiazepam. Peaks: 1 = demoxepam; 2 = oxazepam; 3 = temazepam; 4 = desmethyldiazepam; 5 = diazepam; 6 = desmethylchlordiazepoxide; 7 = chlordiazepoxide.

mixture could be briefly (10 s) centrifuged for better separation of the two layers, although the recovered amount is a decreasing function of exposure time to hexane. Samples for serum drug analysis were identically prepared except drug standards were not added. Instead, serum samples (10–50 μl) were added after the internal standard was initially evaporated to dryness.

RESULTS AND DISCUSSION

Chromatography

Diazepam, chlordiazepoxide and their metabolites are all weakly basic compounds which can be quantitatively extracted into diethyl ether from biological materials buffered to pH 7.0 or greater. Borate buffer (1 *M*, pH 9.0) was chosen over 0.1 *M* sodium hydroxide since it gave cleaner serum blank chromatograms, although others [21, 22] have used 0.1 *M* sodium hydroxide. Fig. 1A shows the chromatogram of a 50-μl serum blank with no apparent peak which would interfere with these benzodiazepines. A chromatogram of a spiked serum sample containing 0.25 μg/ml oxazepam, N-desmethyldiazepam and diazepam and 0.5 μg/ml temazepam is presented in Fig. 1B. Fig. 1C is a representative chromatogram of a rat serum sample obtained 15 min after an intraperitoneal injection of 8 mg/kg diazepam. Using this method we obtained half-lives for oxazepam, temazepam,

TABLE I

PRECISION DATA FOR DIAZEPAM AND IS METABOLITES IN SERUM

Drug	Within-day ($n=5$)		Between-day ($n=5$)	
	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)
Oxazepam	0.050 ± 0.003	6.0	0.051 ± 0.005	9.8
	0.507 ± 0.018	3.6	0.498 ± 0.014	2.8
	1.020 ± 0.055	5.4	1.021 ± 0.034	3.3
Temazepam	0.100 ± 0.007	7.0	0.102 ± 0.007	6.9
	0.764 ± 0.025	3.3	0.762 ± 0.036	4.7
	1.480 ± 0.107	7.2	1.519 ± 0.093	6.1
N-Desmethyldiazepam	0.049 ± 0.002	3.9	0.056 ± 0.004	7.1
	0.508 ± 0.019	3.7	0.512 ± 0.043	8.4
	1.028 ± 0.064	6.2	0.990 ± 0.063	6.4
Diazepam	0.050 ± 0.003	6.0	0.053 ± 0.004	7.5
	0.511 ± 0.023	4.5	0.520 ± 0.044	8.5
	1.011 ± 0.076	7.5	1.001 ± 0.080	8.0

N-desmethyldiazepam and diazepam (unpublished data) that are in close agreement with values reported in the literature [23–26]. The major metabolites of chlordiazepoxide are demoxepam, desmethylchlordiazepoxide, oxazepam and N-desmethyldiazepam [27]. By increasing the methanol concentration in this mobile phase to 55%, all five compounds were resolved (Fig. 1D). Diazepam can be used as an internal standard for the determination of chlordiazepoxide and its metabolites.

Recovery and precision

The recoveries (mean ± S.D.) for oxazepam, temazepam, N-desmethyldiazepam and diazepam were 82.6 ± 0.72, 96.9 ± 5.93, 107 ± 4.6 and 99.9 ± 6.88%, respectively, and were calculated at three concentrations (0.05, 0.25 and 1.0 μg/ml) for these four benzodiazepines.

Within-day and between-day precisions were established on three different concentrations (0.05, 0.5 and 1.0 μg/ml for oxazepam, N-desmethyldiazepam and diazepam; 0.1, 0.75 and 1.5 μg/ml for temazepam) by adding these four compounds to blank serum. The coefficients of variation (C.V.) for these compounds ranged from 3.3 to 7.5% for within-day and 2.8 to 9.8% for between-day precision (see Table I).

Fig. 2 shows that the calibration curves for diazepam and its metabolites are linear within the ranges examined. For each of these four regression lines the correlation coefficients are all greater than 0.99 (Table II). The coefficients of variation of the slopes ($n=6$) of the regression lines ranged from 3.7 to 8.6% with intercepts all close to zero.

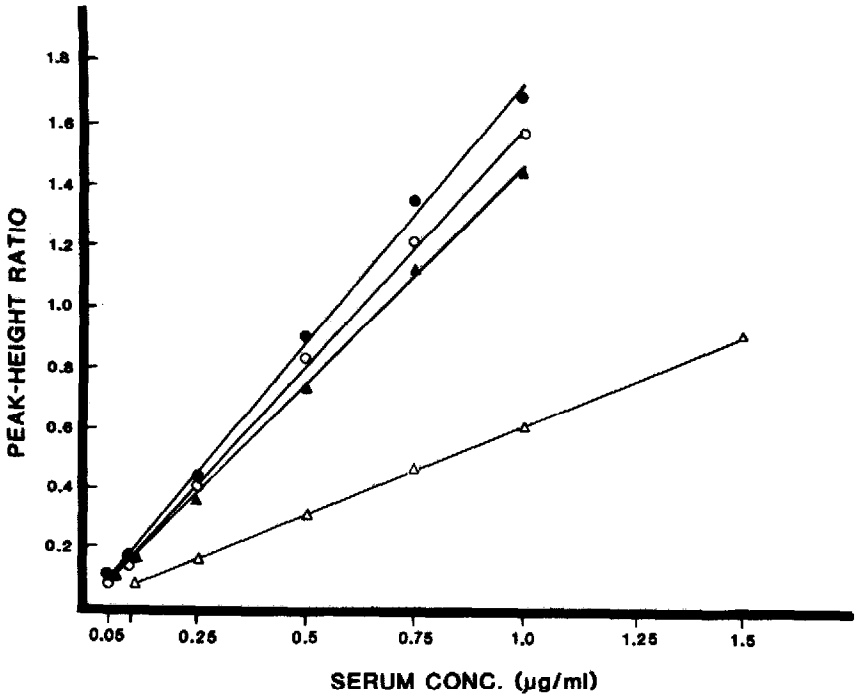

Fig. 2. Calibration curves of oxazepam (○), temazepam (△), N-desmethyldiazepam (●) and diazepam (▲).

Detection and sensitivity

Allowing a signal-to-noise ratio of 25, the limits of detection are 0.25 ng for oxazepam, N-desmethyldiazepam and diazepam and 0.5 ng for temazepam. All four compounds are linear to 10 μg/ml. The lower limit of detection obtained from this method is close to the value reported by Tjaden et al. [13] except that a 1-ml serum sample was required in the latter method and often repeated extraction was needed.

TABLE II

MEAN LINEAR CALIBRATION CURVE FOR THE CONCENTRATION RANGES 0.05–1.0 μg/ml FOR OXAZEPAM, N-DESMETHYLDIAZEPAM AND DIAZEPAM AND 0.1–1.5 μg/ml FOR TEMAZEPAM ESTABLISHED ON SIX DIFFERENT DAYS

y = peak-height ratio (compound/internal standard); x = concentration of each compound.

Drug	Equation	Correlation coefficient	C.V. of slope (%)
Oxazepam	$y=1.5907(\pm0.0593)x+0.0023(\pm0.0192)$	0.998	3.7
Temazepam	$y=0.5943(\pm0.0511)x+0.0087(\pm0.0117)$	0.990	8.6
N-Desmethyldiazepam	$y=1.7417(\pm0.1902)x+0.0115(\pm0.0220)$	0.994	6.3
Diazepam	$y=1.4313(\pm0.1106)x+0.0212(\pm0.0200)$	0.993	7.7

TABLE III

RELATIVE RETENTION TIMES (k') OF SEVERAL BENZODIAZEPINES, THEIR METABOLITES AND SOME OTHER COMMON DRUGS UNDER CHROMATOGRAPHIC CONDITIONS DESCRIBED IN TEXT

N.D. = peak not observed up to 20 min.

Compound	k'	Compound	k'
Caffeine	<2	Temazepam	5.57
Phenobarbital	<2	Chlordesmethyldiazepam	6.02
Barbital	<2	Midazolam	7.29
Demoxepam	2.39	N-Desmethyldiazepam	7.35
Clonazepam	2.80	Haloperidol	8.63
Desmethylchlordiazepoxide	3.31	Diazepam	9.32
Clobazepam	3.85	Reserpine	17.02
Oxazepam	4.40	Chlorpromazine	N.D.
Flurazepam	4.54	Cocaine	N.D.
Chlordiazepoxide	5.20	Methamphetamine	N.D.

Relative retention times

Table III lists the relative retention times (k') of the drugs studied. With the exception of chlordiazepoxide and temazepam, the retention times of the various benzodiazepines are quite different. However, since temazepam is not a metabolite of chlordiazepoxide and it is unlikely that diazepam and chlordiazepoxide will be prescribed concurrently, the co-elution of temazepam and chlordiazepoxide would not be a problem.

Effect of pH

The pH and molarity of the sodium acetate buffer used in the mobile phase are critical for the separation of benzodiazepines. Peaks remained well resolved between pH 3.7 and 4.0. When the pH reached 5.0 or higher, a partial overlap of temazepam and N-desmethyldiazepam peaks was observed.

Effect of wavelength

The absorbance of oxazepam, temazepam, N-desmethyldiazepam and diazepam were determined at various wavelengths between 226 and 254 nm. The maxima were at 228 nm for all four compounds. Similar values were reported for these compounds when they were dissolved in ethanol instead of the mobile phase [28]. The commonly used 254 nm was actually the least sensitive wavelength, averaging 46% less sensitive than 228 nm.

Effect of column packing material

C_{18} 10-μm packing material was used in the early stage of the experiment, however, switching to C_{18} 5-μm packing material resulted in better selectivity and resolution among diazepam, chlordiazepoxide and their metabolites, although midazolam, another benzodiazepine, and its metabolites were much better separated by the C_{18} 10-μm column using the same mobile phase [29].

Taken together, the low detection limit, high precision and relative simplicity of the present method is best suited for the detection of these benzodiazepines in situations where both concentrations of the compounds and size of the sample are extremely limited.

ACKNOWLEDGEMENTS

This work was supported by PHS Grants DA 3117 and AA 00253. The authors are grateful to Dr. John L. Falk, Department to Psychology, Rutgers University, for critical reading of the manuscript.

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