High-Pressure Liquid Chromatographic Determination of Chlordiazepoxide and Its Major Metabolites in Biological Fluids

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Abstract □ A simple, isocratic, reversed-phase, high-pressure liquid chromatographic procedure was developed for the determination of chlordiazepoxide and its major metabolites in plasma and urine. The within-run coefficient of variation was 3.4–8.0%, and the day-to-day variation was 4.0–8.0%. Recoveries of 80–91% with sensitivity limits of 50 ng/ml were obtained for the parent drug and its metabolites. Plasma and urine samples collected after single intravenous and single oral doses were analyzed using this procedure.

Keyphrases □ Chlordiazepoxide—analysis, high-pressure liquid chromatography, biological fluids, metabolites □ Sedatives—chlordiazepoxide, high-pressure liquid chromatographic analysis, biological fluids, metabolites □ High-pressure liquid chromatography—analysis, chlordiazepoxide and metabolites, biological fluids

Chlordiazepoxide hydrochloride (7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepin-4-oxide hydrochloride) (I) was synthesized by Sternbach in 1957 (1) and found to possess pronounced central nervous system activity coupled with low toxicity. At present, I is recommended for the relief of anxiety and tension, withdrawal symptoms associated with acute alcoholism, and preoperative apprehension and anxiety and as an adjunct in the treatment of various disease states in which anxiety and tension are manifest (2).

BACKGROUND

Compound I is metabolized to a number of biotransformation products in humans (3, 4). Of these, *N*-desmethylchlordiazepoxide (II), demoxepam (III), and *N*-desmethyldiazepam (IV) are measurable in plasma after chronic administration. All three compounds exhibit muscle relaxant and anticonvulsant properties in animal species (5, 6) and, therefore, presumably have pharmacological activity in humans.

Various methods have been used for the determination of chlordiazepoxide in blood and other biological fluids, including spectrophotometry (7), colorimetry (8), fluorometry (9, 10), GLC (11–13), and differential pulse polarography (14). All of these procedures have various disadvantages when applied to the analysis of the parent drug and its metabolites.

High-pressure liquid chromatography (HPLC) has been applied to the analysis of the benzodiazepines (15) and also to the quantitation of specific benzodiazepines and their metabolites (16, 17). Recently, the technique was used to quantitate I and its metabolites (18, 19). A gradient elution procedure was described for I and II with chlorpromazine as the internal standard (18), and a reversed-phase column was used to determine I and its metabolites in plasma after oral doses (19).

This report describes a new, simple, isocratic, reversed-phase HPLC procedure that has been used for the analysis of I–III in plasma and urine samples collected after the controlled administration of I to human volunteers.

EXPERIMENTAL

Reagents—Sodium borate[1] (analytical reagent), dibasic sodium phosphate[2] (analytical reagent), methanol[3] (UV grade), and chloroform[3] were used without further purification.

Chlordiazepoxide hydrochloride[4], *N*-desmethylchlordiazepoxide[4], demoxepam[4], and diazepam[4] were used as received.

Apparatus—The modular high-pressure liquid chromatograph consisted of a constant-flow pump[5], a 10-μl loop injector[6], a fixed-wavelength (254 nm) UV detector[7], and a strip-chart recorder[8]. A stainless steel column (4.6 mm × 25 cm) packed with 10-μm silica particles with chemically bonded octadecylsilane[9] was obtained commercially.

Chromatographic Conditions—The mobile phase of methanol–0.025 *M* dibasic sodium phosphate (pH 7.5) (56:44) was used at a flow rate of 1.6 ml/min.

Analytical Procedure—Methanolic solutions (A) corresponding to 1 μg of I, II, or III (free base)/ml were prepared. Solutions of 100 (B) and 10 (C) ng/μl were prepared by serial dilutions of A. All solutions were stored at 0° in the dark. For the quantitation of the drug and its metabolites, standards were prepared by evaporating appropriate amounts of these methanolic solutions to dryness under an air stream at room temperature. Ten milliliters of drug-free plasma or urine was added, and the solutions were shaken for 30 min.

Solutions B and C were prepared every 4 weeks; however, A was stable for a longer period if kept in the dark at 0°. The stability was checked by injecting 1 μg of the drug or metabolite under the described HPLC conditions and comparing the peak height with that obtained initially.

The extraction procedure for plasma consisted of adding 1 μg of diazepam (as the internal standard) and 1 ml of saturated sodium borate (pH 9.0) to 1 ml of plasma. Seven milliliters of chloroform was added, and the mixture was shaken for 10 min. It was centrifuged at 2500 rpm to separate the layers, and the chloroform was transferred to a 15-ml conical tube, where it was evaporated to dryness under an air stream at 60°. The residue was dissolved in 20 μl of the HPLC eluent, and 10 μl was injected. Both the extraction and evaporation tubes had been silanized previously with 10% dimethylchlorosilane[10] in toluene[1].

A similar method was used for the urine samples, except that a 3-ml aliquot was taken and 0.5 μg of the internal standard was used.

Diazepam was chosen as the internal standard because of its similarity in structure and in retention time. Quantitation was achieved by plotting peak height ratio of the drug (or metabolite) to the internal standard against concentration. If the described method is to be used for toxicological screening, a more suitable internal standard would be prazepam, which is not encountered as frequently in toxicology cases.

Table I—Calibration Curve Data ($y = c + mx$)

Sample	Compound	Range, μg/ml	Slope (m)	Intercept (c)	r^a
Plasma	I	0.05–1.0	0.0033	−0.07	0.998
	II	0.05–0.75	0.0044	−0.08	0.996
	III	0.05–0.50	0.005	0.14	0.998
Urine	I	0.025–0.20	0.013	−0.12	0.991
	II	0.025–0.20	0.014	−0.04	0.999
	III	0.025–0.20	0.014	0.072	0.999

[a] Regression coefficient

[1] Mallinckrodt, St. Louis, Mo.

[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] Hoffmann-La Roche, Nutley, N.J.
[5] Model 3200, Spectra-Physics, Santa Clara, Calif.
[6] Valco, Spectra-Physics, Santa Clara, Calif.
[7] Model SP 8200, Spectra-Physics, Santa Clara, Calif.
[8] Model 300, Linear Instruments Corp., Irvine, Calif.
[9] Prepacked Spherisorb ODS, Spectra-Physics, Santa Clara, Calif.
[10] Applied Science Laboratories, State College, Pa.

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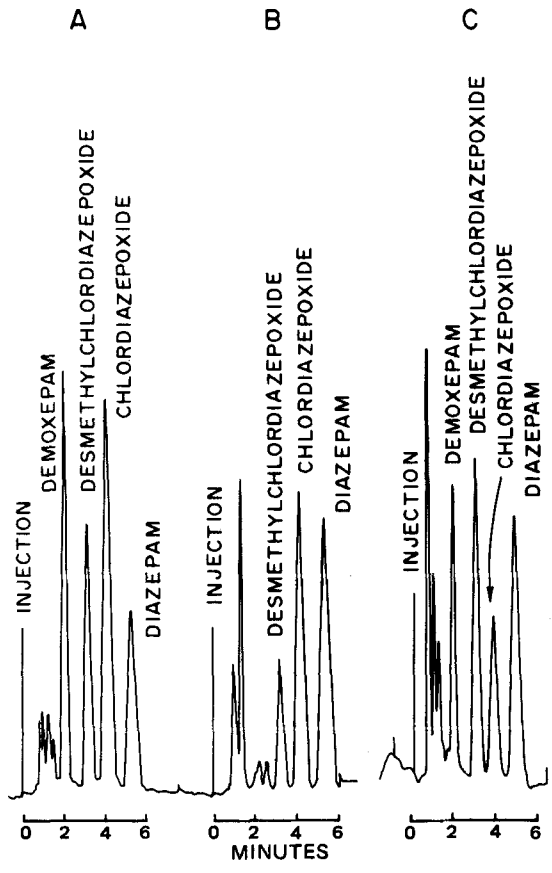

Figure 1—*High-pressure liquid chromatograms of an extracted standard (A), a plasma extract from a patient given 0.12 mg of chlordiazepoxide hydrochloride/kg iv (B), and a plasma extract from a patient on long-term therapy (C).*

RESULTS AND DISCUSSION

The results were linear over the following ranges: I, 50–1000 ng/ml; II, 50–750 ng/ml; and III, 50–600 ng/ml. If the method is to be used at appreciably higher concentrations, two- or fourfold dilution of the sample would be necessary. The equations for the calibration curves and the regression coefficients are listed in Table I. The results of precision studies, both within run and day to day, are shown in Table II.

Recovery studies were performed on I–III using the described conditions. Recoveries were 81 ± 6% of I, 88 ± 5% of II, and 91 ± 4% of III. Sensitivity limits were ~50 ng/ml for each compound using a 1-ml plasma sample per assay. These sensitivity limits possibly could be improved with a detector wavelength of 240 nm.

No interfering peaks were observed on analysis of blank plasma and urine samples. Chromatograms from an extracted standard, a plasma extract from a patient who was given 0.12 mg of I/kg iv, and a plasma extract from a patient on long-term therapy with the drug are shown in Fig. 1.

The described procedure has been used successfully for measuring the drug and its desmethyl metabolite in plasma after oral and intravenous administrations of the parent drug. The plasma concentration–time course curves obtained from four patients were similar to those described by Boxenbaum *et al.* (20).

N-Desmethyldiazepam (IV), a reported metabolite of I, would elute between the parent compound and the internal standard under the described HPLC conditions and have a sensitivity limit of 75 ng/ml. No measurable amounts of IV were seen in plasma samples collected in a 24-hr period following single oral and intravenous doses. This finding is in agreement with that of Dixon *et al.* (4), who found low concentrations (10–60 ng/ml) of IV in plasma samples collected 24–72 hr after a single intravenous dose.

Table II—Statistics for Chlordiazepoxide and Its Metabolites

Compound	Concentration when First Analyzed	Mean	*SD*	*CV*, %
	Within Run (n = 10)			
I	384	383	13	3.4
	220	221	15	6.8
II	271	273	12	4.4
	133	132	9.7	7.4
III	303	307	19	6.3
	117	118	9.4	8.0
	Day to Day (n = 5)			
I	805	750	54.3	7.2
	198	184	7.3	4.0
II	692	666	51.7	7.8
	110	103	5.4	5.2
III	431	424	33.8	8.0
	84	78	3.5	4.5

Urine samples also were analyzed using this method. Demoxepam was the most common metabolite detected, although the percentage of the total dose recovered in the urine as I–III was extremely small (~1% in the first 24 hr after administration).

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