Electron-Capture GLC Determination of Clobazam and Desmethylclobazam in Plasma

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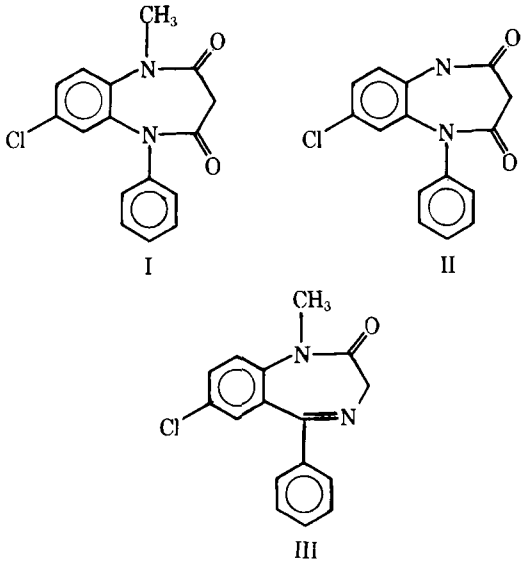
Received November 13, 1979, from the *Division of Clinical Pharmacology, Tufts-New England Medical Center, Boston, MA 02111.* Accepted for publication May 6, 1980.

Abstract □ Electron-capture GLC was utilized for simultaneous quantitation of clobazam, a 1,5-benzodiazepine derivative, and its active metabolite, desmethylclobazam, in human plasma and serum. After addition of diazepam as the internal standard, samples were extracted into benzene–isopentanol. The organic extracts were evaporated to dryness, reconstituted, and subjected to chromatographic analysis without derivatization or cleanup. All three compounds were resolved completely and had favorable chromatographic properties using an OV-101 liquid phase. The limits of detection are 3–5 ng/ml for clobazam and 5–10 ng/ml for desmethylclobazam. Relative standard deviations for identical samples do not exceed 6%. The application of the method to a clinical pharmacokinetic study is demonstrated.

Keyphrases □ Clobazam—electron-capture GLC analysis with desmethylclobazam, human plasma □ Desmethylclobazam—electron-capture GLC analysis with clobazam, human plasma □ GLC, electron capture—analyses, clobazam and desmethylclobazam, human plasma

Clobazam (I) is a 1,5-benzodiazepine derivative utilized as a sedative and antianxiety agent (1–3). The major metabolic pathway of clobazam in humans appears to involve hepatic *N*-demethylation, yielding desmethylclobazam (II) (4). This metabolite also has pharmacological activity (5). The present report describes a rapid and sensitive method for simultaneous quantitation of clobazam and desmethylclobazam in plasma following therapeutic doses of the parent compound.



EXPERIMENTAL

Apparatus and Chromatographic Conditions—The analytical instrument was a gas chromatograph[1] equipped with a 15-mCi ^{63}Ni-electron-capture detector and an electronic integrator. The column was coiled glass (122 cm long × 4 mm i.d.) packed with 10% OV-101 on 80–100-mesh Chromosorb WHP[2]. The carrier gas was argon–methane[3] (95:5) at a flow rate of 25 ml/min. The operating temperatures were: injection port, 310°; column, 265°; and detector, 320°. At the beginning of each workday, the column was primed by injection of 2–3 μl of a solution of azolectin[4] (1 mg/ml) in benzene.

Preparation of Stock Solutions—Clobazam[5] and desmethylclobazam[5] (10 mg) each were dissolved in 2–3 ml of absolute ethanol and diluted to 100 ml with benzene. The stock solution, containing 100 μg/ml, were stored in amber bottles at −4°. Freezing of the stock solutions is recommended because desmethylclobazam may deteriorate when stored unfrozen. Diazepam[6] (III) served as the internal standard in all analyses, and the stock solution of diazepam was prepared similarly.

Working standards, containing 1.0 μg of each compound/ml, were prepared daily by appropriate dilution with benzene or toluene.

Preparation of Samples—A constant amount (100 μl) of a 1-μg/ml working solution of the internal standard was added to round-bottom 13-ml glass culture tubes equipped with polytef-lined screw-top caps. The solutions were evaporated to dryness at 40–50° under mildly reduced pressure in a vacuum oven. Calibration standards were prepared by addition of known amounts of clobazam and desmethylclobazam, ranging from 25 to 300 ng, to some of these tubes. The solvents again were evaporated to dryness.

One milliliter of drug-free control plasma was added to each calibration tube, and 0.5–1 ml of unknown plasma was added to each tube that contained only the internal standard. The calibration standards were extracted and analyzed daily together with each set of unknowns. Samples found to contain clobazam or desmethylclobazam concentrations exceeding the limits of the standard curve were reextracted using a smaller aliquot of plasma.

Extraction—To each tube was added 3–5 ml of benzene (containing 1.5% isopentanol to minimize adherence to glassware). The tubes were agitated for 30 sec on a vortex-type mixer and then centrifuged at room temperature for 10 min at 400×*g*. An aliquot of the organic layer was transferred to another tube and evaporated to dryness at 40° under mildly reduced pressure. The residue was redissolved in 100–200 μl of toluene (containing 15% isopentanol), of which 1–6 μl was injected into the chromatograph.

Clinical Pharmacokinetic Study—A healthy 30-year-old male volunteer participated after giving informed consent. After an overnight fast, a single 20-mg dose of clobazam (two 10-mg tablets) was administered with 150 ml of water. Venous blood samples were drawn into heparinized tubes at multiple time points over the next 168 hr. Plasma was separated and frozen until it was assayed. Plasma concentrations of clobazam and desmethylclobazam were determined as described.

The apparent elimination half-life of clobazam during the terminal (β) phase of the plasma concentration curve was determined by least-squares regression analysis.

RESULTS

Evaluation of Method—Under the described chromatographic conditions, the approximate retention times were: diazepam, 5.5 min; clobazam, 7.4 min; and desmethylclobazam, 8.8 min (Fig. 1).

The relation between the plasma concentration of clobazam or desmethylclobazam and the peak area ratio[7] of each compound to the internal standard was linear through plasma concentrations of at least 300 ng/ml (Table I). The relative standard deviations (*RSD*) for replicate

[1] Hewlett-Packard model 5830A.
[2] Supelco Inc., Bellefonte, Pa.
[3] Matheson Gas Products, Gloucester, Mass.
[4] Associated Concentrates, Woodside, N.Y.
[5] Supplied by Dr. S. K. Puri, Hoechst-Roussel Pharmaceuticals, Somerville, N.J.
[6] Supplied by Dr. W. E. Scott, Hoffmann-La Roche Inc., Nutley, N.J.
[7] Peak height ratio can be used.

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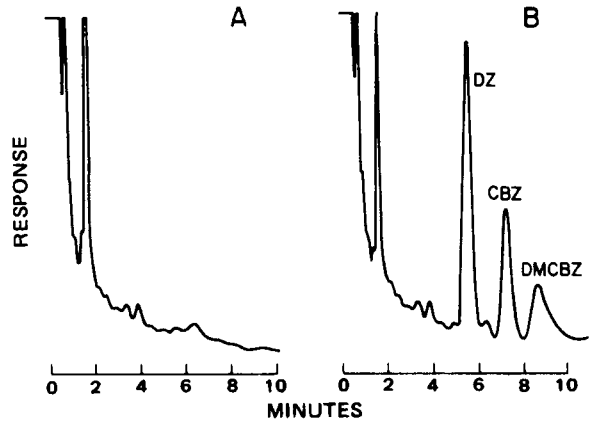

Figure 1—*Chromatogram of a drug-free control plasma extract (A) and chromatogram of the same plasma sample to which was added: diazepam (DZ), 100 ng/ml; clobazam (CBZ), 50 ng/ml; and desmethylclobazam (DMCBZ), 50 ng/ml (B). The chromatographic conditions are given in the text.*

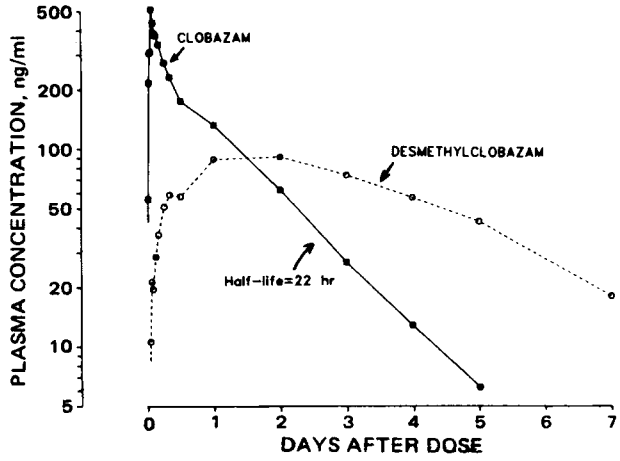

Figure 2—*Plasma concentrations of clobazam and desmethylclobazam over 168 hr after a single 20-mg oral dose of clobazam administered to a healthy male volunteer.*

samples (n = 10) containing 25 ng of clobazam and desmethylclobazam/ml were 1.8 and 5.4%, respectively. At 50 ng/ml, the *RSD* values were 2.5 and 2.9%. The approximate detection limits were 3–5 ng/ml of original sample for clobazam and 5–10 ng/ml of original sample for desmethylclobazam; below these limits, the *RSD* values exceeded 10%. Residue analysis indicated that recovery of clobazam, desmethylclobazam, and diazepam was >95% complete and independent of concentration.

Plasma samples from patients receiving clobazam are stable at room temperature for at least 24 hr. Freezing is recommended for prolonged storage. Plasma extracts also are stable at room temperature and can be stored frozen after reconstitution. Samples are not sensitive to ambient light or to temperatures of <40°.

Pharmacokinetic Study—A peak plasma clobazam concentration of 510 ng/ml was reached at 1.5 hr after dosage (Fig. 2). The elimination half-life during the terminal (β) phase of the curve was 22 hr. Disappearance of clobazam was mirrored by formation of its metabolic product. The highest desmethylclobazam concentration was 91 ng/ml, which was reached 48 hr after clobazam dosage.

DISCUSSION

The sensitivity and specificity of the electron-capture detector have facilitated development of quantitative assays for a number of benzodiazepines in human blood and plasma following therapeutic doses (6–10). The present report describes the application of this approach to the simultaneous quantitation of clobazam and its major pharmacologically active metabolite, desmethylclobazam. Preliminary studies in this laboratory suggested that phenyl methyl silicone liquid phases (such as OV-17) could be used for this purpose. However, chromatographic peaks for the desmethyl metabolite were asymmetric, thereby complicating reliable quantitation. Utilization of a less polar silicone phase (OV-101) substantially improves the chromatographic properties of desmethylclobazam but still allows complete resolution of clobazam and its metabolite from the internal standard and from endogenous plasma contaminants, provided a relatively high phase load (10%) is used.

The clinical pharmacokinetic study illustrates that the method is suitable for pharmacokinetic studies of clobazam and its metabolite following single therapeutic doses. Absorption of clobazam following oral dosage in the fasting state was rapid, with the peak plasma concentration attained 1.5 hr after administration. Clobazam elimination proceeded with a half-life of 22 hr and was mirrored by the formation of desmethylclobazam, which, in turn, was eliminated more slowly than the parent drug. Since both compounds have pharmacological activity, plasma concentrations of both compounds must be measured simultaneously and considered to elucidate the clinical implications of the kinetic properties of clobazam.

Table I—Representative Calibration Data

Concentration, ng/ml	Area Ratio	
	Clobazam	Desmethylclobazam
25	0.17	0.18
50	0.33	0.35
75	0.54	0.56
100	0.70	0.74
150	1.07	1.14
200	1.44	1.56
300	2.12	2.35
Correlation coefficient	0.999	0.999
Slope (±*SE*)	0.00714 (±0.000066)	0.00795 (±0.0000607)
Intercept (±*SE*)	−0.009 (±0.01)	−0.039 (±0.01)
Slope through origin (±*SE*)	0.00710 (±0.000036)	0.00774 (±0.000066)

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ACKNOWLEDGMENTS

Supported in part by Grant MH-12279 from the U.S. Public Health Service, by Grant 77-611 from the Foundations' Fund for Research in Psychiatry, and by a grant-in-aid from Hoechst-Roussel Pharmaceuticals, Somerville, N.J.

The author thanks Ann Locniskar, Marcia Divoll Allen, Ann Werner, Dr. Richard I. Shader, Dr. Surendra K. Puri, and Dr. Dean S. MacLaughlin for assistance. Dr. MacLaughlin was supported by Grant GM-23430 from the U.S. Public Health Service to the Boston Collaborative Drug Surveillance Program.