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Fluorometric Determination of Clobazam, a 1,5-Benzodiazepine, in Human Plasma

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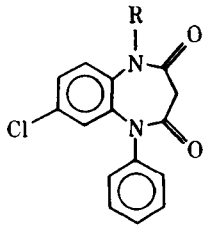
Received April 26, 1978, from the **Bioavailability Group Studies, School of Pharmacy, University of Georgia, Athens, GA 30602*, and ‡*Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ 08876.* Accepted for publication September 25, 1978. §Present address: Pharma-Forschung, Hoechst, A.G., Frankfurt, West Germany.

Abstract □ A fluorometric procedure for clobazam, a 1,5-benzodiazepine, based on a fluorophore formed upon irradiation of the drug using short wavelength UV light (254 nm) for 35 min is presented. Fluorescence is linear over a 100–6400-ng/ml range using excitation and emission wavelengths of 350 and 400 nm, respectively. Application of the method to the determination of clobazam in spiked human plasma samples revealed that the drug can be determined at nanogram per milliliter levels with an accuracy of 1–5%. The procedure is specific for clobazam in samples containing its major plasma metabolite, *N*-desmethylclobazam, and also in samples containing 1,4-benzodiazepines and other selected drugs. A plasma level–time profile after oral administration of a single 40-mg dose of clobazam to a healthy adult male is also illustrated.

Keyphrases □ Clobazam—fluorometric determination in human plasma □ Benzodiazepines—fluorometric determination of clobazam in human plasma □ Tranquilizers—clobazam, fluorometric determination in human plasma □ Fluorometry—analysis, clobazam in human plasma

Clobazam (I) [7-chloro-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione] is a new antianxiety agent currently under clinical investigation. It has demonstrated relatively low sedation potential (1, 2) and relatively limited effects on normal levels of human performance[1] (3, 4). An effective therapeutic dose of 20–40 mg/day has been suggested (4).



I: R = CH_3
II: R = H

[1] J. A. Kotzan, T. E. Needham, I. L. Honigberg, J. J. Vallner, J. T. Stewart, W. J. Brown, and H. W. Jun, presented at the APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1978.

Table I—Typical Calibration Data for Clobazam in Human Plasma

Initial Concentration, ng/ml	Fluorescence Intensity[a]	Slope	Intercept	$r \pm s_{y \cdot x}$ [b]
150	4.05 ± 0.07	0.0258	0.2525	0.9998 ± 0.0906
300	8.00 ± 0.01			
600	15.75 ± 0.21			

[a] Based on duplicate samples. [b] s is the standard error of estimate of y (fluorescence intensity) on x (concentration).

This paper presents a fluorometric procedure for the analysis of clobazam in human plasma. The method allows the determination of drug in the presence of *N*-desmethylclobazam (II), its major plasma metabolite, which has also been reported to possess psychosedative and anticonvulsant activities with low toxicity (5). The utilization of fluorescence offers nanogram per milliliter sensitivity along with suitable reproducibility and accuracy.

EXPERIMENTAL

Apparatus and Reagents—Fluorescence measurements were obtained using a spectrophotofluorometer[2] equipped with a corrected spectra accessory and operated in the true emission mode. Excitation and emission slits were set at 7 and 4 nm, respectively, and sample sensitivity for all measurements was set at 1.

Clobazam powder[3] was used for the preparation of a stock solution (1 μg/ml) in plasma. This solution was prepared by the addition of 0.25 ml of an ethanolic clobazam solution (200 μg/ml) to a 50-ml volumetric flask, followed by the addition of the blank human plasma[4] to volume. *N*-Desmethylclobazam, mp 305–307°, was synthesized according to the procedure of Rossi *et al.* (6). All other chemicals were the highest purity available from commercial sources and were utilized as received.

Determination of Calibration Curve—Quantities of 0.15, 0.30, and 0.60 ml of the clobazam stock solution in plasma were each placed in 15-ml ground-glass stoppered centrifuge tubes, followed by the addition of blank human plasma to make 1 ml. The solution was mixed[5] for 1 min.

[2] Model MPF-4, Perkin-Elmer, Norwalk, Conn.
[3] Hoechst-Roussel Pharmaceuticals, Somerville, NJ 08876.
[4] Obtained from a local hospital blood bank.
[5] Vortex-Genie mixer, Scientific Industries, Bohemia, N.Y.

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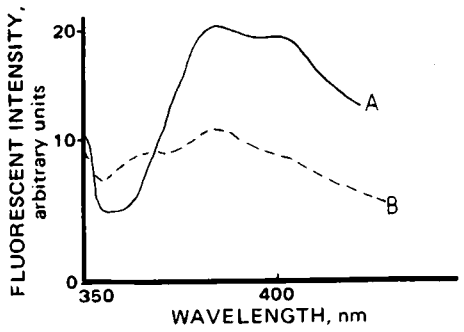

Figure 1—*True emission spectra of clobazam in ethanol before (- -B) and after (—A) irradiation (254 nm) for 35 min. Excitation wavelength was 350 nm.*

Hexane (5 ml) was then added, and the two phases were mixed[5] for an additional 30 sec. After centrifugation for 1 min at 4000 rpm, 4 ml of the hexane phase was transferred to a clean 15-ml centrifuge tube and evaporated to dryness in a water bath (80 ± 1°) using a nitrogen stream.

The residue was then redissolved in 1 ml of ethanol, and a portion of the solution was transferred to a 0.5-ml quartz microcell[6] (pathlength of 5 mm). A fluorometric measurement was obtained using excitation and emission wavelengths of 350 and 400 nm, respectively. The solution was then evenly irradiated at close proximity to the microcell with two short wavelength UV lamps[7] for 35 min, followed by fluorescence measurement at the same excitation and emission wavelengths (E_2).

The final fluorescence value (E) was calculated from $E = E_2 - E_1$. The three concentrations of clobazam used in the calibration curves were then subjected to linear regression analysis; the slope, intercept, and regression coefficient were calculated as shown in Table I.

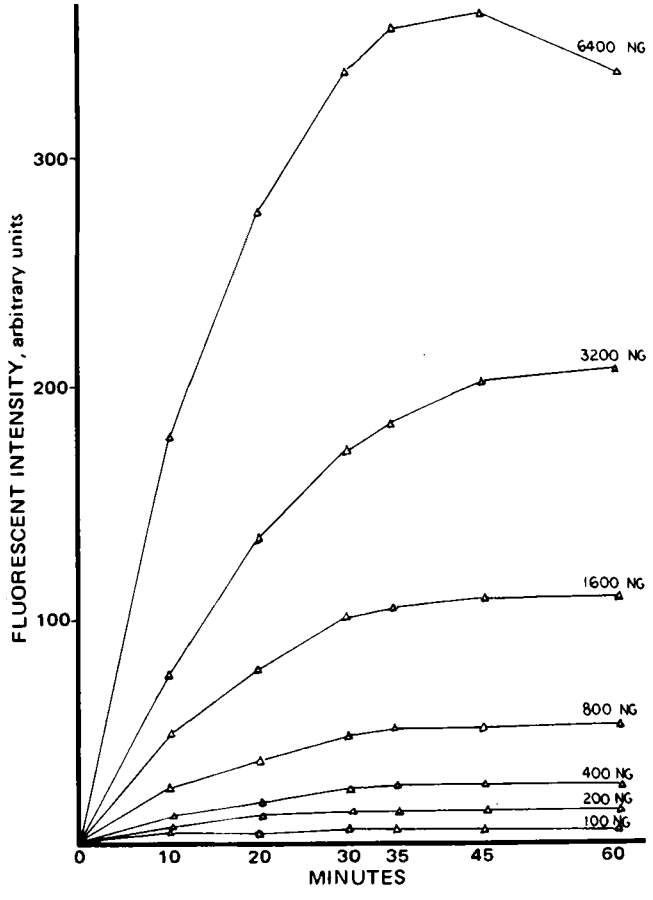

Figure 2—*Plot of fluorescent intensity of clobazam at various concentrations in ethanol after irradiation (254 nm) of the ethanolic solutions for different time periods.*

[6] Part 018-0056, Hitachi Ltd., Tokyo, Japan.
[7] Model UVS-11, Ultraviolet Products, San Gabriel, Calif.

Determination of Clobazam in Human Plasma—A 1.0-ml sample of human plasma containing clobazam was placed in a 15-ml ground-glass stoppered centrifuge tube and treated in the same manner as described under *Determination of Calibration Curve*, starting with: "Hexane (5 ml) was added . . . ." A calibration curve was run concurrently. Slope and intercept data obtained from the regression analysis of the blank and standard plasma samples were utilized to solve for drug concentration in the plasma samples: [fluorescence intensity = (slope × concentration) + intercept][8].

RESULTS AND DISCUSSION

Interest in the development of a procedure to measure therapeutic plasma clobazam levels for a bioavailability study led to the use of fluorescence. The fluorometric procedure for clobazam reported here is based on a fluorophore, formed when an ethanolic solution of the drug is irradiated with short wavelength UV light (254 nm) (7). Examination of corrected emission spectra before and after irradiation (Fig. 1) revealed that the irradiated solution was more fluorescent than the nonirradiated solution at emission wavelengths greater than 370 nm, with maximum intensity occurring around 400 nm. Use of this wavelength in the true emission mode of the spectrophotofluorometer required an excitation wavelength of 350 nm.

A study of fluorescence intensity *versus* irradiation time for varying concentrations of clobazam (Fig. 2) revealed that a 35-min irradiation time was satisfactory for the assay procedure, especially for the anticipated plasma drug levels (100–1000 ng/ml) after a single 20–40-mg dose (4, 8). Drug concentrations in the range of 1000–6400 ng/ml (Fig. 2) would require slightly longer irradiation times to reach the plateau region of the irradiation curve. However, with the 35-min irradiation time, fluorescence was linear over the 100–6400-ng/ml concentration range of clobazam with $r = 0.9995$. The minimum detection limit for the drug is 50 ng/ml (signal/noise = 2) using the calibration curve from a series of spiked plasma samples.

Studies to determine the nature of the fluorophore formed upon drug irradiation are underway. The desmethyl metabolite forms a fluorophore with a similar emission spectrum under the conditions used for clobazam.

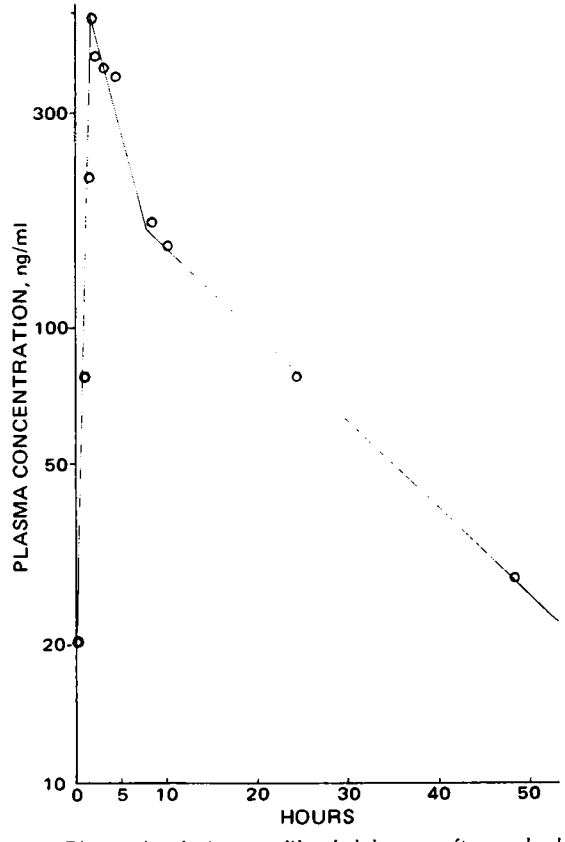

Figure 3—*Plasma level–time profile of clobazam after oral administration of a 40-mg dose to a healthy adult male.*

[8] Olivetti-Underwood programma 101.

Table II—Analysis of Spiked Plasma Samples for Clobazam

Mixture	Concentration Added, ng/ml	Concentration Found[a], ng/ml	Accuracy, %
1 Clobazam	200	196.90 ± 2.30	1.55
2 Clobazam	400	409.75 ± 15.00	2.44
3 Clobazam	600	608.00 ± 28.00	1.33
4 Clobazam	1200	1211.75 ± 29.81	0.98
5 Clobazam	300	285.45 ± 6.43	4.85
N-Desmethylclobazam	300		
6 Clobazam	600	604.00 ± 23.00	0.67
N-Desmethylclobazam	600		
7 Clobazam	400	389.30 ± 2.20	2.68
Diazepam[b]	400		
8 Clobazam	400	373.35 ± 11.35	6.66
Chlordiazepoxide hydrochloride[c]	400		
9 Clobazam	400	368.85 ± 6.85	7.79
Oxazepam[d]	400		
10 Clobazam	400	398.00 ± 29.00	0.50
Clidinium bromide[e]	400		
11 Clobazam	400	376.00 ± 20.00	6.00
Aspirin	400		

[a] Based on duplicate samples. [b] Valium, Hoffmann-La Roche, Nutley, N.J. [c] Librium, Hoffmann-La Roche, Nutley, N.J. [d] Serax, Wyeth Laboratories, Radnor, Pa. [e] Quarzan, Hoffmann-La Roche, Nutley, N.J.

The other known metabolites of clobazam, in which hydroxy and hydroxy and methoxy substituents are present on the drug molecule, have not been shown in these laboratories to form fluorophores and should not interfere with the analysis of clobazam.

Initial extractions of clobazam from spiked plasma samples were performed using ether (9) (percent extraction = 85.58 ± 7.59%). Analysis of a 1-ml plasma sample spiked with 400 ng each of clobazam and *N*-desmethylclobazam with ether as the extraction solvent indicated that the clobazam concentration was 608.95 ± 19.05 ng/ml (n = 2). Assay of a plasma sample containing only the metabolite (400 ng/ml) gave 215.40 ± 0.40 ng/ml (n = 2), expressed as clobazam concentration. These data establish that the fluorophore formed from the desmethyl metabolite interferes with the clobazam analysis because of concurrent extraction of drug and metabolite from the plasma sample. Thus, partitioning studies were performed to find an organic solvent that would selectively extract clobazam from the desmethyl compound.

Chloroform, benzene, hexane, cyclohexane, petroleum ether, heptane, carbon tetrachloride, methylene chloride, and ethyl acetate were investigated. Hexane provided the best selectivity for the extraction of clobazam over that of the *N*-desmethyl metabolite at equimolar concentrations. The percent extraction of clobazam from plasma with hexane was 51.27 ± 2.00%.

Application of the fluorometric assay to the determination of spiked plasma samples containing clobazam and to plasma samples containing mixtures of clobazam and *N*-desmethylclobazam, some 1,4-benzodiazepines, or other drugs is presented in Table II. Determination of clobazam in these samples was achieved with accuracy in the range of 0.50–7.8%.

Figure 3 shows a plasma level–time profile after oral administration of a single 40-mg dose of clobazam to a healthy adult male. The data, obtained by using the described fluorometric procedure, indicate that the method should be applicable to studies where plasma levels of the intact drug are desired.

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Effect of *N*-Trifluoroacetyl Derivatives of Amino Acids and Amino Acid Analogs on Microbial Antitumor Screen

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Received August 11, 1978, from the *Laboratory of Pathophysiology, National Cancer Institute, Bethesda, MD 20014.* Accepted for publication September 28, 1978.

Abstract □ Eighteen trifluoroacetyl derivatives of amino acids and of amino acid analogs were prepared and tested for growth-inhibitory activity using a *Lactobacillus casei* system as a prescreen for antitumor activity. Of the compounds tested, the trifluoroacetyl derivatives of *o*-, *m*-, and *p*-fluorophenylalanine and of β-3-thienylalanine showed modest activity; trifluoroacetyl derivatives of phenylalanine and of β-2-thienylalanine showed marginal activity. The activity exhibited by the active trifluoroacetyl compounds was equal to that noted for most active chloroacetyl derivatives reported previously, as judged by comparison of their activity with that of chloroacetyl-*m*-fluorophenylalanine. No reversal of inhibition was noted when a representative of these inhibitors was challenged with a corresponding natural metabolite, both as a free amino acid and as a noninhibitory acylated compound.

Keyphrases □ Amino acids and analogs—*N*-trifluoroacetyl derivatives, effect on microbial antitumor screen □ Antitumor activity—18 trifluoroacetyl derivatives of amino acids and amino acid analogs, growth inhibitory activity using *Lactobacillus casei* system

An alteration in the biological behavior of certain amino acids, both the naturally occurring and the "unnatural," as measured by growth-inhibitory capacity in a microbial system, was noted upon acylation (1–3). The extent of such activity varied with the nature of the acyl group and the amino acid moiety (3). While *N*-acetyl and *N*-propionyl derivatives of these amino acids showed no appreciable growth-inhibitory activity, the *N*-chloroacetyl derivatives showed modest activity (3), particularly with those amino acids that are normally considered to be essential for

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