Quantitative Determination of Chlordiazepoxide and Its Metabolites in Serum by Fluorescence TLC–Densitometry

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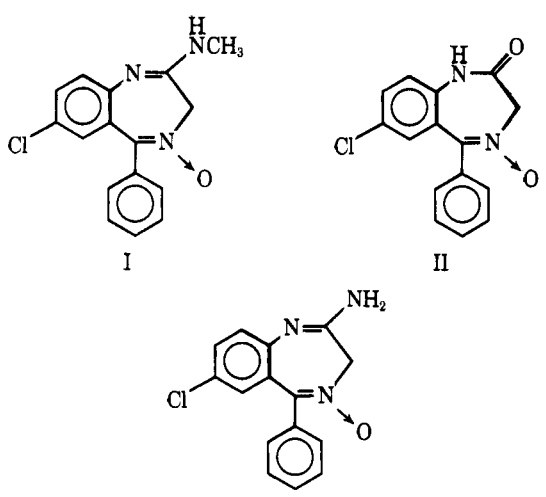
Abstract □ A sensitive fluorescence TLC–densitometric procedure was developed for the specific determination of chlordiazepoxide (I) and its two metabolites, demoxepam (II) and desmethylchlordiazepoxide (III), in serum. After extraction from serum with ether, I, II, and III were separated by TLC and converted with a sulfuric acid spray to pale blue (R_f 0.63), green (R_f 0.54), and blue (R_f 0.45) fluorescence spots, respectively. Quantitation was accomplished by scanning the plate with a densitometer at 390 (I), 430 (II), and 390 (III) nm. The sensitivities were 0.05 (I), 0.01 (II), and 0.01 (III) μg/ml of serum. The procedure was successfully applied to measurement of I–III in human serum after oral administration of 20 mg of chlordiazepoxide hydrochloride.

Keyphrases □ Chlordiazepoxide—and metabolites, fluorescence TLC–densitometric analyses in serum □ Fluorescence TLC–densitometry—analyses, chlordiazepoxide and metabolites in serum □ Densitometry–fluorescence TLC—analyses, chlordiazepoxide and metabolites in serum □ Sedatives—chlordiazepoxide and metabolites, fluorescence TLC–densitometric analyses in serum

The determination of chlordiazepoxide (I) and its metabolite demoxepam (II) in plasma was reported previously (1). It was later modified (2) to include another metabolite, desmethylchlordiazepoxide (III). This determination is based on the quantitative conversion of I and III, by controlled hydrolytic conditions, to II. Compound II is converted, with light, to a compound exhibiting a characteristic fluorescence in alkaline solution, which is measured spectrofluorometrically.

The assay of I is the least accurate of the three since it involves the subtraction of one fluorescence (I and III) from another (III). The reported sensitivity is about 0.2–~0.3 μg/ml for each compound. Schwartz *et al.* (3) later increased the sensitivity to 0.1 μg/ml by using a spectrofluorometer containing a new xenon lamp. However, their procedure is not sensitive enough to measure demoxepam levels in any chlordiazepoxide-treated subject (3).



I

II

III

The present TLC–densitometric procedure is for the simultaneous specific determination of I–III in serum. The sensitivities for I, II, and III are 0.05, 0.01, and 0.01 μg/ml of serum, respectively, which are sufficient to monitor the blood levels of each compound in a chlordiazepoxide bioavailability study.

EXPERIMENTAL

Reagents and Materials—Chlordiazepoxide hydrochloride[1], demoxepam[2], and desmethylchlordiazepoxide[3] were used as supplied. Ether[4] was anesthetic grade, and the other chemicals were analytical reagent grade. TLC plates[5] (20 × 20 cm) were used as supplied.

Apparatus—A two-speed reciprocating shaker[6] and a refrigerated centrifuge[7] were used. Sample solutions in the test tubes were mixed[8] prior to analysis. Samples were spotted on TLC plates with an automatic TLC spotter[9] using polytef-tipped 50-μl syringes[10]. A TLC sprayer[11] was used to apply the sulfuric acid spray on the plates.

Preparation of Serum Standards—Five serum standards were extracted for each eight unknown samples. Serum standards contained I, II, and III at 1.5, 0.3, and 0.3 μg/ml, respectively, in the first standard; 1.0, 0.2, and 0.2 μg/ml, respectively, in the second standard; 0.5, 0.1, and 0.1 μg/ml, respectively, in the third standard; and 0.2, 0.04, and 0.04 μg/ml, respectively, in the fourth standard. The fifth standard was the serum blank[12].

The first serum standard was prepared by spiking the serum blank[12] with the following primary standard solutions: I hydrochloride, 100 μg/ml in water; II, 100 μg/ml in methanol; and III, 100 μg/ml in methanol.

Extraction Procedure—To 2 ml of serum sample or standard in a 20-ml screw-capped test tube were added 2 ml of 0.1 *M* K_2HPO_4 and 5 ml of ether. The tube was shaken reciprocally at 80 cpm for 5 min. After centrifugation at 3000 rpm for 5 min at 10°, 4 ml of the ether layer was immediately transferred to a 15-ml conical test tube and evaporated to dryness at 40° with filtered air. The residue was reconstituted with 1 ml of ether and dried again at 40° with filtered air. To the dry residue was added 50 μl of methanol, and the contents were mixed. The entire amount was taken up in a 50-μl syringe and spotted on a TLC plate using an automatic TLC spotter. The heating region of the automatic spotter was set at 35–40°.

Thirteen samples (eight unknowns and five standards) were spotted on each TLC plate. A horizontal line was scribed 2.5 cm from the top of the TLC plate, and the plate was developed to the line with chloroform–methanol–ammonium hydroxide (85:15:0.5). After the plate was dried overnight at room temperature, it was sprayed about six times with 7 *M* H_2SO_4. The spots were located with long wavelength UV light[13]. The compounds appeared as pale-blue (R_f 0.63, I), green (R_f 0.54, II), and blue (R_f 0.45, III) fluorescence spots on the TLC plate.

[1] 7-Chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepin-4-oxide hydrochloride, Abbott internal reference standard, A-21064, lot 351074, Abbott Laboratories, North Chicago, Ill.

[2] 7-Chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one-4-oxide, NF reference standard, lot 69145.

[3] 7-Chloro-2-amino-5-phenyl-3*H*-1,4-benzodiazepin-4-oxide, Abbott internal reference standard, A-47203, Abbott Laboratories, North Chicago, Ill.

[4] Mallinckrodt Chemicals, St. Louis, Mo.

[5] Silica gel GF, 250 μm, Analtech, Inc., Newark, Del.

[6] Eberbach Corp., Ann Arbor, Mich.

[7] Sorvall Inc., Newtown, Conn.

[8] Vortex Genie, model K-550-GT, Scientific Industries, Springfield, Mass.

[9] Analytical Instrument Specialties, Libertyville, Ill.

[10] Scientific Products, McGaw Park, Ill.

[11] Sprayon power unit, Sprayon Products, Cleveland, Ohio.

[12] K-N Enterprises, Inc., Skokie, Ill.

[13] UV Viewbox, Ultra-Violet Products, San Gabriel, Calif.

Table I—Assay Reproducibility

Compound	Theoretical	Serum Concentration, μg/ml[a] Observed Run 1	Run 2	Run 3	Mean ± SD	Relative SD, %
I[b]	0.20	0.23	0.27	0.21	0.237 ± 0.031	13.1
	0.50	0.44	0.47	0.43	0.447 ± 0.021	4.7
	1.00	0.99	1.00	1.02	1.000 ± 0.015	1.5
	1.50	1.39	1.57	1.51	1.490 ± 0.092	6.2
II[c]	0.04	0.044	0.044	0.041	0.043 ± 0.0017	4.0
	0.10	0.098	0.106	0.091	0.098 ± 0.0075	7.7
	0.20	0.182	0.209	0.197	0.196 ± 0.0135	6.9
	0.30	0.284	0.308	0.316	0.303 ± 0.0167	5.5
III[d]	0.04	0.044	0.047	0.041	0.044 ± 0.0030	6.8
	0.10	0.096	0.099	0.099	0.098 ± 0.0017	1.8
	0.20	0.186	0.198	0.195	0.193 ± 0.0062	3.2
	0.30	0.291	0.308	0.314	0.304 ± 0.0119	3.9

[a] All 12 serum samples were on a single TLC plate. [b] R_f 0.63. [c] R_f 0.54. [d] R_f 0.45.

Densitometry—The TLC plate was scanned at 30 mm/min, in a direction perpendicular to the direction of development, with a densitometer[14] fitted with a suitable barrier filter in the compartment of the measuring head. The TLC plate was irradiated in the reflectance mode, using a xenon lamp as the light source, at the following maximum excitation wavelengths: I, barrier filter FL 43, λ_{max} 390 nm; II, barrier filter FL 46, λ_{max} 430 nm; and III, barrier filter FL 43, λ_{max} 390 nm.

The slit width was 0.5 × 12 mm. The chart speed of the recorder was 10 mm/min. The high voltage potential on the photomultiplier tube was set at 1, and the gain was set at 20 on the arbitrary scale of the instrument[15]. Each spot on the TLC plate was manually located to give maximum response.

RESULTS AND DISCUSSION

The excitation spectra for the fluorescence spots are shown in Fig. 1. These spectra were constructed by plotting the relative fluorescence intensity (as measured by the peak heights) at different excitation wavelengths.

The reproducibility of the assay is shown in Table I. Relative standard deviations in a triplicate study for I, II, and III ranged from 1.5 to 13.1%, from 5.5 to 7.7%, and from 1.8 to 6.8%, respectively, indicating good reproducibility.

Recoveries were 99.0 ± 1.2% (mean ± *SD*) for triplicate I serum samples at 1.5 μg/ml, 78.6 ± 4.5% for triplicate II serum samples at 0.3 μg/ml, and 83.6 ± 3.2% for triplicate III serum samples at 0.3 μg/ml. These values were calculated by comparing the peak height responses between the extracted serum samples and the theoretical amount of pure drug spotted on the TLC plate. TLC was used to achieve specificity (Fig. 2).

The sensitivity limits of the assay were about 0.05, 0.01, and 0.01 μg/ml of serum for I, II, and III, respectively (Table II). These values were calculated by fitting a regression line to the standard curve and taking the 95% confidence interval of the *y*-intercept (in peak height). The calculated sensitivity was the *x*-value (in concentration) corresponding to the upper part of this interval of the *y*-intercept. In calculating the sensitivity for I, the linear portion of the curve (the first four points only) was used.

The accuracy of the assay was tested by determining seven unknown spiked serum samples. The unknown serum concentrations were calculated from the standard curves obtained from the five serum standards

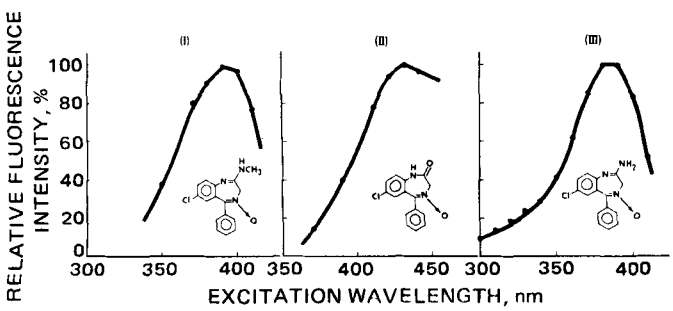


Figure 1—*Fluorescence excitation spectra of I–III.*

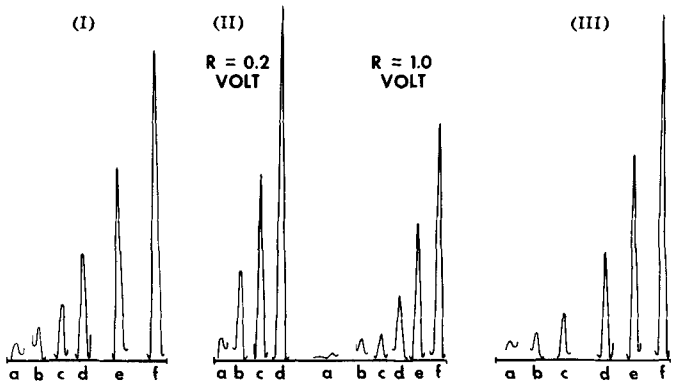


Figure 2—*TLC-densitometric tracings of serum extracts containing I–III. Key: a, serum blank; b, serum standard containing 0.05 μg of I/ml, 0.01 μg of II/ml, and 0.01 μg of III/ml; c, serum standard containing 0.10 μg of I/ml, 0.02 μg of II/ml, and 0.02 μg of III/ml; d, serum standard containing 0.20 μg of I/ml, 0.04 μg of II/ml, and 0.04 μg of III/ml; e, serum standard containing 0.50 μg of I/ml, 0.10 μg of II/ml, and 0.10 μg of III/ml; and f, serum standard containing 1.00 μg of I/ml, 0.20 μg of II/ml, and 0.20 μg of III/ml.*

extracted and spotted on the same TLC plate. The results (Table III) indicated good accuracy. This precision was due in part to the use of an automatic TLC spotter. This device allowed the simultaneous uniform spotting of all 13 samples within 15 min.

The developed method was used to measure serum concentrations of I–III in normal human subjects after oral administration of two 10-mg chlordiazepoxide hydrochloride capsules[16] in a single dose. The subjects were fasted for 12 hr prior to dosing. The drug was administered with 120 ml (4 oz) of water.

Typical serum concentration *versus* time curves for two human subjects are shown in Fig. 3. Peak serum chlordiazepoxide concentrations occurred at 40 min and were 1.16 and 1.10 μg/ml. Serum levels presisted to 32 hr (the final sampling time). The 0–32-hr *AUC*'s (areas under the curve) calculated by the trapezoidal rule were 5.73 and 8.59 μg × hr/ml. The serum chlordiazepoxide half-lives estimated from the terminal linear log concentration *versus* time plots were 10.3 and 9.5 hr. These values agreed with the ranges reported earlier (3–6). Serum II and III levels appeared soon after administration of the parent drug, and II levels slowly increased throughout the duration of blood sampling. The *AUC*'s for III for the two subjects were 3.78 and 6.35 μg × hr/ml, and the *AUC*'s for II were 1.49 and 1.34 μg × hr/ml.

The results indicate that the present method can be employed to quantitate serum I–III levels in a single-dose chlordiazepoxide bioavailability study. Differential spectrofluorometric methods (2, 3) generally used for chlordiazepoxide bioavailability studies (5–9) had a limit of sensitivity of about 0.1–0.2 μg/ml for I–III using 2-ml specimens. In all of these single-dose studies (3, 5, 6), plasma II levels were less than 0.2 μg/ml.

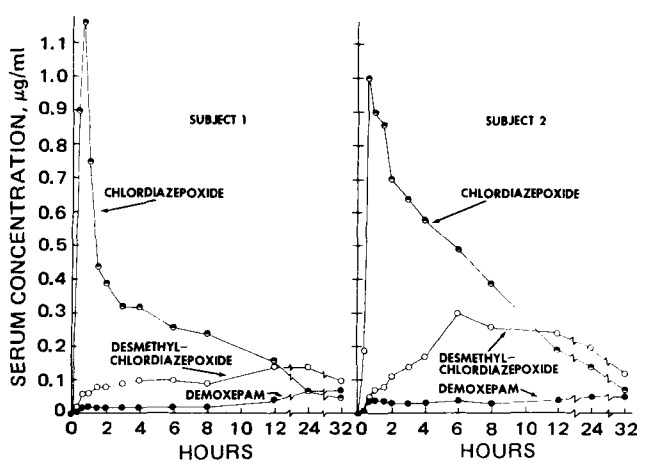


Figure 3—*Serum I–III levels of two individuals after 20 mg po of chlordiazepoxide hydrochloride capsules.*

[14] Zeiss PMQ II chromatogram spectrophotodensitometer, Carl Zeiss, Oberkochen, West Germany.

[15] Zeiss PMI indicator.

[16] Librium, Roche, lot 3672-02135.

Table II—Assay Sensitivity

Serum I Concentration, μg/ml	Peak Height, mm, R = 0.5 v[a]	Serum II Concentration, μg/ml	Peak Height, mm		Serum III Concentration, μg/ml	Peak Height, mm	
			R = 1 v	R = 0.2 v[a]		R = 1 v[a]	R = 0.2 v[a]
0	6	0	1.5	7	0	3	27
0.05[b]	11	0.01[b]	6	35	0.01[b]	8.5	64
0.10[b]	21	0.02[b]	11	75	0.02[b]	17.5	134
0.20	43	0.04	20	142	0.04	43	
0.50	77	0.10	55		0.10	81.5	
1.00	125	0.20	96		0.20	139	
1.50	156	0.30	139		0.30	196	
Least-Squares Linear Regression Calculation[c]							
Number of points	4 (first four samples)		7			7	
Slope	190.28		462.19			643.16	
y-Intercept ± 95% confidence limit	3.600 ± 9.026		2.690 ± 4.095			8.225 ± 9.664	
Correlation coefficient	0.991		0.999			0.996	
Calculated sensitivity, μg/ml[d]	0.0474		0.0089			0.0150	

[a] Recorder full-scale deflection. [b] Included here to determine the sensitivity. It was not included in the normal standards. [c] Reference 13. [d] The calculated sensitivity was the x-value (in concentration) corresponding to the upper part of the 95% confidence interval of the y-intercept (in response).

Table III—Accuracy of the Assay

Unknown Serum Sample Number	Serum Concentration, μg/ml								
	I			II			III		
	Theoretical	Calculated	% Difference	Theoretical	Calculated	% Difference	Theoretical	Calculated	% Difference
1	0.20	0.21	5.0	0.04	0.05	25.0	0.04	0.04	0
2	0.50	0.58	16.0	0.10	0.11	10.0	0.10	0.13	30.0
3	1.00	1.06	6.0	0.20	0.23	15.0	0.20	0.22	10.0
4	1.20	1.23	2.5	0.24	0.27	12.5	0.24	0.26	8.3
5	0.75	0.65	13.3	0.15	0.18	20.0	0.15	0.16	6.2
6	0.10	0.09	10.0	0.02	0.02	0	0.02	0.02	0
7	0.05	0.04	20.0	0.01	0.01	0	0.01	0.01	0
		Average	10.4		Average	11.8		Average	7.8

A specific spectrofluorometric determination of chlordiazepoxide in biological fluids with fluorescamine was reported previously (10). However, II and III could not be measured by this procedure. Zingales (11) reported the determination of chlordiazepoxide in plasma by an electron-capture GLC procedure that could not determine the metabolites. A thorough review of the determination of some 1,4-benzodiazepines and their metabolites in body fluids was described by Clifford and Smyth (12).

Strong fluorescence characteristics with a sulfuric acid spray on TLC plates are also observed for other 1,4-benzodiazepines such as diazepam, desmethyldiazepam, oxazepam, 4′-hydroxydesmethyldiazepam, and nitrazepam. A similar quantitative fluorescence–TLC method should be applicable for these compounds.

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