Fluorescence–TLC Densitometric Determination of Diazepam and Other 1,4-Benzodiazepines in Serum

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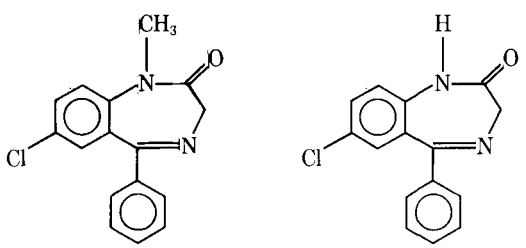
Abstract □ A sensitive fluorescence–TLC densitometric procedure was developed for the specific determination of diazepam (I) and its two metabolites, desmethyldiazepam (II) and oxazepam (III), in serum. After extraction from serum with benzene, the compounds were separated by TLC and converted with a sulfuric acid spray to greenish-yellow fluorescence spots with R_f values of 0.72, 0.43, and 0.17, respectively. Quantitation of the TLC plate was accomplished by scanning with a densitometer at 380 nm. The sensitivities of the assay were 18 (I), 6 (II), and 5 (III) ng/ml of serum. This procedure also was applicable to other 1,4-benzodiazepines in biological fluids.

Keyphrases □ Diazepam—fluorescence–TLC densitometric analysis in serum □ Desmethyldiazepam—fluorescence–TLC densitometric analysis in serum □ Oxazepam—fluorescence–TLC densitometric analysis in serum □ Fluorescence–TLC densitometry—analyses, diazepam, desmethyldiazepam, and oxazepam in serum □ Sedatives—diazepam, fluorescence–TLC densitometric analysis in serum

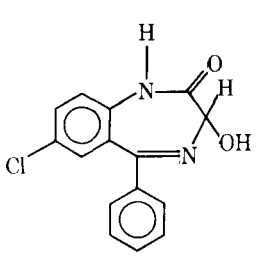
Diazepam and its metabolites have been determined in biological fluids primarily by electron-capture GLC (1–9). Clifford and Smyth (10) reviewed the determination of some 1,4-benzodiazepines and their metabolites in body fluids by different instrumental analyses. Other investigators (11, 12) studied the fluorescence properties of some 1,4-benzodiazepines in acidic solutions. Recently, TLC procedures for the determination of diazepam (I) and its metabolites, desmethyldiazepam (II) and oxazepam (III), were reported (13–16). Measurements were taken directly from an unstained thin-layer plate to diffuse-light reflectance of I–III at their UV absorption maxima.

The following new fluorescence–TLC method is capable of measuring low levels of I–III in serum. Compounds I–III are converted to greenish-yellow fluorescence spots on TLC plates by a sulfuric acid spray and measured densitometrically. A similar fluorescence technique for the determination of chlordiazepoxide and its metabolites in serum was reported (17). Strojny *et al.* (16) also examined several fluorescent derivatives by TLC densitometry, but their procedure was less sensitive because of the use of a second monochromator rather than a filter as in the present study.



I

II



III

EXPERIMENTAL

Reagents and Materials—Diazepam[1] (I), desmethyldiazepam[2] (II), and oxazepam[3] (III) were used as supplied. The other chemicals were analytical reagent grade. TLC plates[4] (20 × 20 cm) were used as supplied.

Apparatus—A two-speed reciprocating shaker[5] and a refrigerated centrifuge[6] were used. Sample solutions in the test tubes were mixed[7]. Samples were spotted on TLC plates with an automatic TLC spotter[8] using polytef-tipped 50-μl syringes[9]. A TLC sprayer[10] was used for 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 0.60, 0.20, and 0.20 μg/ml, respectively, in the first standard; 0.30, 0.10, and 0.10 μg/ml, respectively, in the second standard; 0.15, 0.05, and 0.05 μg/ml, respectively, in the third standard; and 0.075, 0.025, and 0.025 μg/ml, respectively, in the fourth standard. The fifth standard was the serum blank[11]. The first serum standard was prepared by spiking the serum blank with primary standard solutions of I–III each at 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 1 ml of 0.1 *M* K_2HPO_4 and 5 ml of benzene. The tube was shaken reciprocally at 80 cpm for 10 min. After centrifugation at 3000 rpm for 5 min at 10°, 4 ml of the benzene layer was 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 methanol and dried at 40° with filtered air; then 50 μl of methanol was added and 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 plate, and the plate was developed to the line with chloroform–2-propanol–ammonium hydroxide (95:5:0.5).

After the plate was dried overnight at room temperature, it was stood vertically and sprayed about six times with 7 *N* H_2SO_4. The acid was sprayed across the plate at a distance of about 15.2 cm (6 in.) from it and at the rate of about 5 sec/spray. The spots were located with long wavelength UV light[12]. The compounds appeared as greenish-yellow fluorescence spots with R_f values of 0.72, 0.43, and 0.17 for I, II, and III, respectively.

Densitometry—The TLC plate was scanned at 100 mm/min in a direction perpendicular to the direction of development with a densitom-

[1] 7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, lot 309039, Hoffmann–La Roche, Nutley, N.J.
[2] 7-Chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one, lot 101-294, Hoffmann–La Roche, Nutley, N.J.
[3] 7-Chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one, Wyeth, Philadelphia, Pa.
[4] Silica gel GF (250 μm), Analtech, Newark, Del.
[5] Eberbach Corp., Ann Arbor, Mich.
[6] Sorvall Inc., Newtown, Conn.
[7] Vortex Genie, model K-55-GT, Scientific Industries, Springfield, Mass.
[8] Analytical Instrument Specialties, Libertyville, Ill.
[9] Scientific Products, McGaw Park, Ill.
[10] Sprayon power unit, Sprayon Products, Cleveland, Ohio.
[11] K-N Enterprises Inc., Skokie, Ill.
[12] UV Viewbox, Ultra-Violet Products, San Gabriel, Calif.

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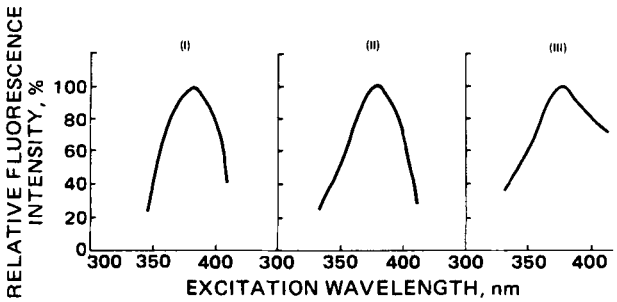

Figure 1—*Fluorescence excitation spectra of I–III.*

eter[13] fitted with a barrier filter (FL 43) 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 maximum excitation wavelength of 380 nm. (See *Results and Discussion.*) 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 2, and the gain was set at 20 on the arbitrary scale of the instrument[14]. Each spot on the TLC plate was manually located to give maximum response.

RESULTS AND DISCUSSION

The excitation spectra for the fluorescence spots (Fig. 1) were constructed by plotting the relative fluorescence intensity (as measured by the peak heights) at different excitation wavelengths. The maximum excitation wavelength was 380 nm for I–III. This wavelength was used for the assay.

Reproducibility of the assay was tested by assaying serum samples from 0.05 to 0.60 μg/ml. Relative standard deviations in a triplicate study on a single TLC plate for I, II, and III ranged from 1.1 to 15.1, from 2.0 to 6.3, and from 9.1 to 12.2%, respectively. Assay of triplicate serum samples on three TLC plates showed relative standard deviations of 0.8–20.2, 9.8–12.4, and 2.4–19.9% for I, II, and III, respectively, indicating larger variabilities among plates. However, the serum standards must be extracted and spotted along with the unknowns on each TLC plate.

Recoveries were 96.4 ± 0.7% (mean ± *SD*) for triplicate I serum samples at 0.6 μg/ml, 87.8 ± 4.3% for triplicate II serum samples at 0.2 μg/ml, and 87.3 ± 3.9% for triplicate III serum samples at 0.2 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. The R_f values for I, II, and III were 0.72, 0.43, and 0.17, respectively. Typical TLC densitometric tracings are shown in Fig. 2.

The sensitivity limits of the assay were about 18, 6, and 5 ng/ml of serum for I, II, and III, respectively. 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 diazepam, 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 sample. The unknown serum concentrations were calculated from the standard curves obtained from the five serum standards extracted and spotted on the same TLC plate. The average percent differences between the observed and theoretical concentrations for serum samples from 0.025 to 0.600 μg/ml were 4.2, 4.3, and 5.6% for I, II, and III, respectively, indicating good accuracy. This precision was partly due to the use of an automatic TLC spotter. This device allowed the simultaneous uniform spotting of all 13 samples within 15 min.

Assay of control serum sample from a commercial source[11] showed a small background peak in the area of each compound (Fig. 2). Therefore, serum samples obtained from volunteers were assayed and compared. The background peaks from different subjects varied and generally were smaller than the background peak of the commercial source[11]. Thus, to compensate for the background variability, the concentration from 0 hr was subtracted from all values in the calculation.

This analytical method was used for the measurement of serum diazepam and metabolite concentrations in normal human subjects after

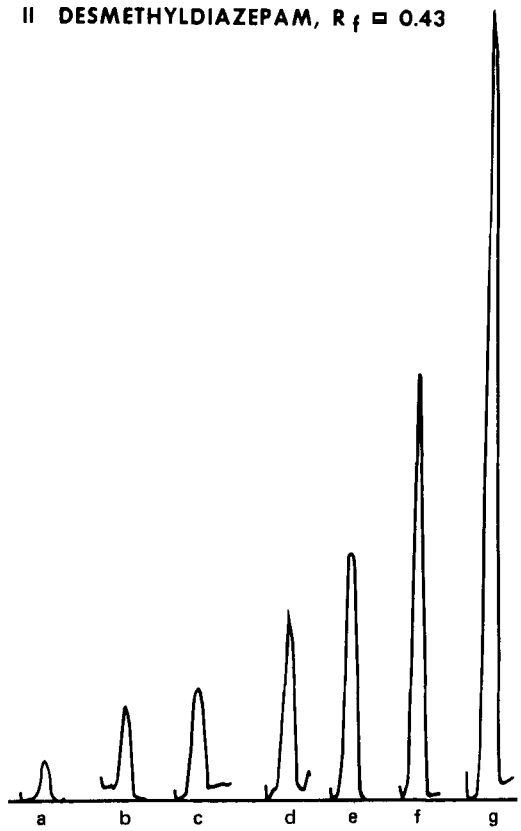

Figure 2—*TLC densitometric tracings of serum extracts containing I–III. Key: a, serum blank; b, serum standard containing 0.019 μg of I/ml, 0.006 μg of II/ml, and 0.006 μg of III/ml; c, serum standard containing 0.038 μg of I/ml, 0.012 μg of II/ml, and 0.012 μg of III/ml; d, serum standard containing 0.075 μg of I/ml, 0.025 μg of II/ml, and 0.025 μg of III/ml; e, serum standard containing 0.15 μg of I/ml, 0.05 μg of II/ml, and 0.05 μg of III/ml; f, serum standard containing 0.30 μg of I/ml, 0.10 μg of II/ml, and 0.10 μg of III/ml; and g, serum standard containing 0.60 μg of I/ml, 0.20 μg of II/ml, and 0.20 μg of III/ml.*

oral administration of diazepam tablets[15] in a single 10-mg dose. The subjects were fasted for 12 hr prior to dosing. The drug was administered with 120 ml (4 oz) of water. A serum concentration–time curve for one subject is shown in Fig. 3. The peak serum diazepam concentration occurred at 20 min and was 0.47 μg/ml. Desmethyldiazepam levels slowly increased throughout the duration of blood sampling. These values agreed with the ranges reported earlier (18). Oxazepam was not detected in this subject.

The results of this study indicate that the developed fluorescence–TLC method can be employed to quantitate diazepam and its metabolite (desmethyldiazepam) serum levels in a single-dose diazepam bioavail-

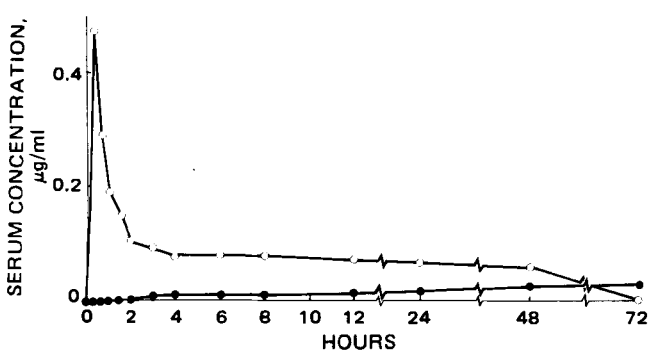

Figure 3—*Serum levels of I (○) and II (●) in one subject after receiving a 10-mg oral dose of diazepam.*

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

[15] Valium, Roche, 5 mg/tablet, lot 6259-04165.

ability study. These fluorescent characteristics by a sulfuric acid spray on the TLC plate also were observed for other 1,4-benzodiazepines and were applied for the determination of chlordiazepoxide and its metabolites in serum (17). When chloroform–2-propanol–ammonium hydroxide (95:5:0.5) is used for separation, some 1,4-benzodiazepines have different fluorescence colors under long wavelength UV light after a sulfuric acid spray with R_f values of 0.32 (chlordiazepoxide, blue fluorescence), 0.19 (demoxepam, yellowish green), 0.38 (nitrazepam, blue), 0.73 (medazepam, blue), 0.74 (prazepam, blue), and 0.06 (*p*-hydroxydesmethyldiazepam, yellow). If chloroform–methanol–acetic acid (85:15:1) is used, these compounds have similar fluorescence characteristics after a sulfuric acid spray and their R_f values are 0.79 (diazepam), 0.73 (desmethyldiazepam), 0.65 (oxazepam), 0.68 (chlordiazepoxide), 0.66 (demoxepam), 0.72 (nitrazepam), 0.77 (major spot of medazepam which has a very small spot at R_f 0.14), 0.79 (prazepam), and 0.56 (*p*-hydroxydesmethyldiazepam). By the combined use of these solvent systems and the characteristic fluorescence colors, this procedure should be applicable for the general screening test of some 1,4-benzodiazepines in both serum and urine samples.

REFERENCES

(1) J. A. F. de Silva, M. A. Schwartz, V. Stefanovic, J. Kaplan, and L. D'Arconte, *Anal. Chem.*, **36**, 2099 (1964).

(2) J. A. F. de Silva, B. A. Koechlin, and G. Bader, *J. Pharm. Sci.*, **55**, 692 (1966).

(3) J. A. F. de Silva and C. V. Puglisi, *Anal. Chem.*, **42**, 1725 (1970).

(4) F. Marcuss, R. Fonelli, and E. Mussini, *J. Chromatogr.*, **37**, 318 (1968).

(5) A. Berlin, B. Siwers, S. Agurell, A. Hiort, F. Sjögvist, and S. Ström, *Clin. Pharmacol. Ther.*, **13**, 733 (1972).

(6) R. Erkkola, L. Kangas, and A. Pekkarinen, *Acta Obstet. Gynecol. Scand.*, **52**, 167 (1973).

(7) L. Kangas, A. Pekkarinen, C. Sourander, and E. Raijola, *Ann. Clin. Res., Suppl. 11*, **6**, 12 (1974).

(8) J. Hendel, *Acta Pharmacol. Toxicol.*, **37**, 17 (1975).

(9) J. A. F. de Silva, I. Bekersky, C. V. Puglisi, M. A. Brooks, and R. E. Weinfeld, *Anal. Chem.*, **48**, 10 (1976).

(10) J. M. Clifford and W. F. Smyth, *Analyst*, **99**, 241 (1974).

(11) J. Braun, G. Caille, and E. A. Martin, *Can. J. Pharm. Sci.*, **3**, 65 (1968).

(12) G. Caille, J. Braun, D. Gravel, and R. Plourde, *ibid.*, **8**, 42 (1973).

(13) N. Wad, H. Rosenmund, and E. Hanifl, *J. Chromatogr.*, **128**, 231 (1976).

(14) N. Wad and E. Hanifl, *ibid.*, **143**, 219 (1977).

(15) S. Ebel and H. Kusmaul, *Arch. Pharm.*, **307**, 230 (1974).

(16) N. Strojny, K. Bratin, M. A. Brooks, and J. A. F. de Silva, *J. Chromatogr.*, **143**, 363 (1977).

(17) S. Sun, "APhA Annual Meeting Abstract," vol. 7, No. 1, American Pharmaceutical Association, Washington, D.C., 1977, p. 194. S. Sun, *J. Pharm. Sci.*, **67**, 639 (1978).

(18) S. A. Kaplan, M. L. Jack, K. Alexander, and R. E. Weinfeld, *J. Pharm. Sci.*, **62**, 1789 (1973).

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Facile Separation of Sulfonamides from Their Degradates by Liquid–Liquid Extraction

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Abstract □ Regulation of acidity for protonation of the free N^4-amine can provide for the selective liquid–liquid extraction isolation of a sulfonamide from its degradation products. This principle is applied for the stability-indicating determination of sulfacetamide in the presence of sulfanilamide, sulfaquinoxaline in feed, and sulfabromomethazine in dosage forms. In solution, sulfabromomethazine can exhibit photodecomposition to sulfamethazine. The mean relative errors of the these methods and the precision, represented by relative standard deviations, are each typically <2%.

Keyphrases □ Sulfonamides, various—separated from degradation products by liquid–liquid extraction □ Degradation products of various sulfonamides—separated from parent compounds by liquid–liquid extraction □ Liquid–liquid extraction—separation of various sulfonamides from degradation products □ Antibacterials—various sulfonamides, separation from degradation products by liquid–liquid extraction

Although most sulfonamides are highly stable, they can degrade (almost exclusively) by acid-catalyzed hydrolysis of the sulfur–nitrogen bond (1–7) or, occasionally, by cleavage of the nitrogen–carbon bond (1). Hence, any stability-indicating analysis for these drugs in formulations should discriminate between the intact sulfonamide and both sulfanilic acid and sulfanilamide as potential hydrolytic degradates.

BACKGROUND

Applications of separation techniques to the sulfonamides have long been of interest because of the similar chemical and physical properties of these drugs and their use in combinations. Typically, sulfonamides have been separated by TLC, and many very exacting quantitative procedures for determining mixtures of these drugs using this technique have been published. Among those studies that present data for sulfanilamide (*e.g.*, 8, 9) are evaluations of new spray reagents (10, 11) and various stationary phases (12).

Of the GLC methods, those procedures that measure the relevant amine generated from acidic sulfonamide hydrolysis (4–7) are not directly applicable for stability studies; only the methods that derivatize the intact drug (13–18) are appropriate. Some recently published high-pressure liquid chromatographic (HPLC) sulfonamide separations make use of adsorption (19, 20), ion-exchange (21, 22), ion-pair partition (23, 24), and reversed-phase (25) modes.

For stability studies of sulfonamides, simple liquid–liquid extraction is an attractive alternative to some of those tedious or time-consuming approaches. In particular, quantitative TLC methods are lengthy and require careful attention to technique to obtain good precision. The extraction is also convenient because it neither requires the derivatization essential for GLC nor relies on instrumentation, and it can be combined with standard (*e.g.*, Bratton–Marshall) analytical measurements for the sulfonamides. Although other solvents also may be satisfactory, a mixed chloroform–1% dimethylformamide solvent was selected as the organic phase to demonstrate the feasibility of this approach. The aqueous phase

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