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ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Allied Health and Industrial Sciences, College of Pharmacy and Allied Health Professions, St. John's University, Jamaica, NY 11439*

The assistance of Carmen Barreto in the literature search and of Karen Mertens and Jean Cerruti in the preparation of the manuscript is gratefully acknowledged.

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RESEARCH ARTICLES

Spectrofluorodensitometric Determination of Flurazepam and Its Major Metabolites in Blood

J. ARTHUR F. de SILVA ˣ, IHOR BEKERSKY, and CARL V. PUGLISI

Abstract □ The spectrofluorodensitometric assay for the determination of flurazepam and its major metabolites in blood involves selective extraction of flurazepam and its major metabolites into ether and hydrolysis in acid to their respective benzophenones, which are cyclized in dimethylformamide–potassium carbonate to their respective 9-acridanone derivatives and separated by TLC. The fluorescence of the 9-acridanones is measured directly on the chromatoplate, using a scanning TLC analyzer in the reflectance mode. The limits of detection of the assay are 0.5–1.0 ng of each compound/ml of blood using a 4-ml specimen/analysis.

Keyphrases □ Flurazepam and major metabolites—fluorometric TLC analysis in blood □ TLC, spectrofluorodensitometry—analysis, flurazepam and metabolites in blood □ Spectrofluorodensitometry—analysis, flurazepam and metabolites

The quantitation of organic compounds separated by TLC has usually been done after elution of the component into a suitable solvent, followed by the use of an appropriate physicochemical assay procedure (1). These methods impart a high degree of flexibility and specificity for quantitation. However, the precision of the assay requires minimal physical loss of the silica gel during its transfer from the chromatoplate and maximal elution recovery of the compound.

Direct quantitation of compounds on a chromatoplate using scanning chromatogram analyzers has the distinct advantages of greater precision, sensitivity, and time saving (2–5). The advantages and disadvantages of quantitative measurements made in the transmission *versus* the reflectance modes of operation for spectrophotodensitometry and spectrofluorodensitometry have been extensively discussed (6–13). The use of these direct scanning techniques for the quantitation of drugs has also been well documented (11–16).

A spectrofluorometric assay for the determination of flurazepam[1] (I) and its major metabolites in blood and urine, the hydroxyethyl (II) and *N*-desalkyl (III) analogs, was reported (17), which uses the fluorescence of their respective 9-acridanone derivatives (18, 19) for quantitation after TLC separation. The chemical structures and reactions of flurazepam (I) and its major metabolites are shown in Scheme I.

The use of a direct scanning chromatogram analyzer enabled the simultaneous quantitation of the 9-acridanones with greater precision and sensitivity because the component to be quantitated is concentrated over a small surface area. The three compounds are completely resolved from each other and from interfering biological contaminants and migrate as distinct fluorescent spots (Fig. 1). They are quantitated by scanning the chromatoplate at a fixed excitation wavelength (383 nm) and measuring the fluorescence emitted (at 457 nm) at a 45° angle of reflectance (20).

[1] Flurazepam, 7-chloro-1-[2-(diethylamino)ethyl]-5-(*o*-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one dihydrochloride, is the active drug in the pharmaceutical formulation Dalmane, Hoffmann-La Roche Inc., Nutley, N.J.

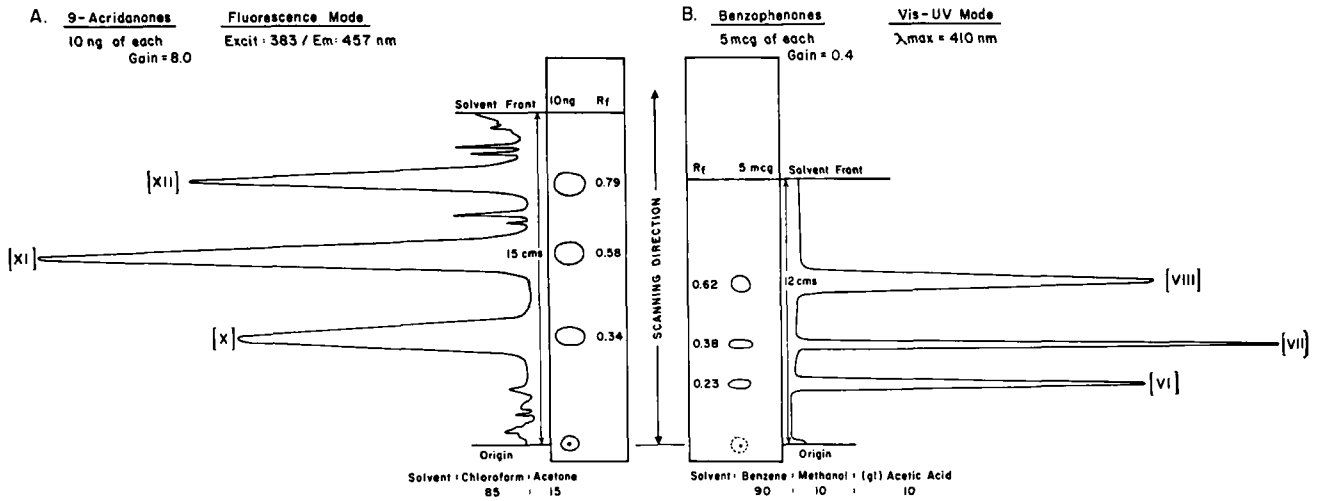


Figure 1—*Thin-layer chromatograms and densitometric scans of: (A) the 9-acridanones determined in the fluorescence mode, and (B) the benzophenones determined in the visual–UV mode.*

The limits of detection are 0.5–1.0 ng each of I, II, and III per milliliter of blood using a 4-ml specimen/analysis.

Blood levels of flurazepam and its major metabolites were determined in two subjects following a single 30-mg dose of flurazepam administered orally.

EXPERIMENTAL

Instrumentation—A TLC spectrophotodensitometer[2] equipped with a 150-w, dc xenon arc energy source was used for direct scanning in the 45° reflectance mode (20). The instrument is equipped with motor-driven dual monochromators for excitation and emission measurements and for automatic wavelength scanning of the excitation and emission spectra of a component directly on the chromatoplate.

The following instrumental parameters were used for the measurement of fluorescence emission of the 9-acridanones.

Fluorescence Mode—The excitation monochromator was set at 383 nm; the exciter leg (beam condenser) has a 0.500-in. reducer (spacer) and a No. 7-54 optical filter. The emission or analyzer monochromator was set at 457 nm, and a No. 3-73 optical filter was used with the 300–800-nm transmission lens in the analyzer leg without any reducers. A sensitive photomultiplier[3] was used in conjunction with 10-nm slits at the analyzer (Table I).

The chromatoplate was scanned spatially at a rate of 4 in./min in the single-beam recording mode, with the recorder chart speed also set at 4 in./min. A baseline controller connected between the electrometer output and the recorder was essential for adjusting the baseline above electrical zero for accurate quantitation. This adapter was retrofitted to this instrument to correct a drift problem.

Calibration—Authentic standards of known concentrations of the three acridanones were spotted as standard mixtures on the plate and developed on the same plate as the biological specimens to minimize the plate variables (Fig. 1A). These standards were used to calibrate the response of the instrument and to adjust it according to the expected concentration range of the unknowns.

Absorption (Visual-UV) Mode—This mode is proposed for the analysis of the benzophenone (VII) produced by hydrolysis of the major metabolite (II), present in urine as a glucuronide conjugate. Although the benzophenones are intensely yellow and can be quantitated at 400 nm, greater sensitivity can be achieved by using the major UV band at 258 nm where the absorbance is about 4–5 times that at 400 nm (17). In this mode, the amount of the excitation energy reflected from the surface of the spot (*i.e.*, not absorbed by the compound) is measured at a 45° angle.

For this purpose, the emission monochromator was removed and the photomultiplier housing, containing a UV-sensitive photomultiplier tube[4], was installed in its place. The excitation monochromator was set at 258 nm, and this energy was incident upon the plate through the beam condenser lens (200–375 nm) in the exciter leg containing a 0.625-in. reducer (spacer) and no optical filters. The emission monochromator was removed and the analyzer leg was used with the 200–350-nm lens, in conjunction with a 0.125-in. reducer and a No. 7-54 optical filter. A 10-nm entrance slit was used with the 1P-28 photomultiplier (Table I).

Chromatoplates—Precoated 20 × 20-cm silica gel G chromatoplates of 60-μm particle size and 250-μm bed thickness were used[5]. The samples were applied to the origin of the chromatogram using calibrated 50-μl disposable pipets.

Analysis of Blood Specimens by Fluorodensitometry of Acridanones—The blood specimens are processed exactly as previously described (17), along with a set of standards of the authentic compounds I, II, and III added to blood as a mixture covering the concentration range of 10, 50, or 100 ng of each compound. These standards are used for the determination of each compound in the unknowns. Mixtures (10, 50, or 100 ng) of authentic standards of the 9-acridanones X, XI, and XII are applied to the chromatoplate as the reference standards for calibrating the instrument and as markers for locating the 9-acridanones in the biological extracts.

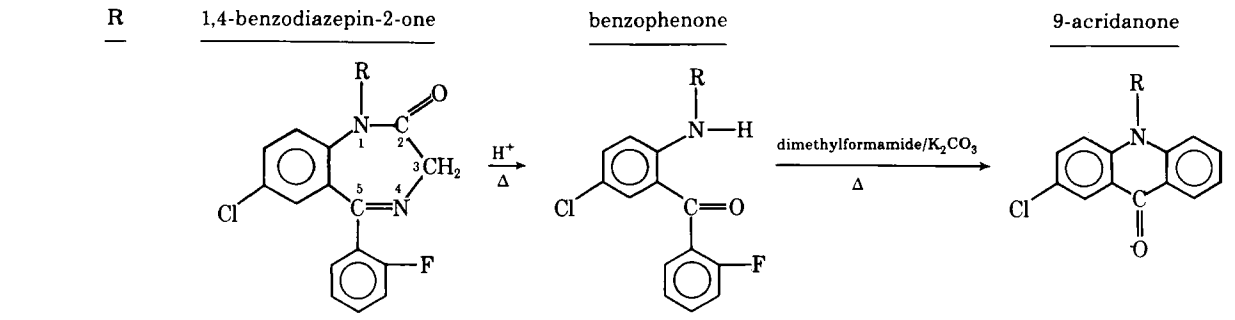
The residues containing the 9-acridanones from the blood extracts are dissolved in 100 μl of chloroform, transferred onto 20 × 20-cm precoated silica gel G thin-layer chromatoplates, and developed in chloroform–acetone (85:15) as described (17) to separate the three 9-acridanones X, XI, and XII (Scheme I). The plate is air dried and examined under short- and longwave UV light, and the respective 9-acridanones (X, R_f 0.34; XI, R_f 0.58; and XII, R_f 0.79) (Fig. 1A) are located visually as intensely fluorescent blue-green spots by reference to the R_f values of the 100-ng mixture of the authentic acridanone standards run alongside the sample extracts.

Since the intrinsic fluorescence of each 9-acridanone is approximately equal, the amplifier gain is set to give 95–98% full-scale deflection on the recorder pen for the 100-ng standard of any one of the acridanones and then scanned to check the fluorescence emission of the other two for full-scale pen response. The chromatoplate is scanned in the fluorescence mode described and the fluorescence emission of each compound is recorded as symmetrical Gaussian-shaped peaks (Fig. 1A). The peak area [peak height (centimeters) × width (centimeters) at half-height], which is determined using either the slope baseline technique or electronic digital integrations, is proportional to concentration (nanograms) of the acridanone.

[2] Model 137190 visual-UV chromatogram analyzer, Farrand Optical Co., Mount Vernon, N.Y.

[3] The RCA 1P-21 photomultiplier used is a specially selected tube (purchased from Farrand) for maximum sensitivity.

[4] RCA 1P-28 or equivalent photomultiplier tube.



R	1,4-benzodiazepin-2-one	benzophenone	9-acridanone
$-(CH_2)_2N(C_2H_5)_2$	I	VI	X
$-CH_2CH_2OH$	II	VII	XI
$-H$	III	VIII	XII
$-H$	IV, 3>CHOH	VIII	XII
$-CH_2COOH$	V	IX	XIII

Scheme I—Chemical reactions of N-1-alkyl-substituted o-fluoro-1,4-benzodiazepin-2-ones (see Ref. 17)

Table I—Instrumental Parameters for the Operation of the Visual–UV TLC Analyzer[a]

Function		Excitation System			Detection System				
		Excitation–Beam–Condenser			Analyzer–Draw		Detector		
Mode	Excitation Wavelength, nm	Lens, nm	Reducer	Optical Filter	Reducer	Optical Filter	Exit Slit, nm	Emission Wavelength, nm	Photomultiplier
Fluorescence	383	375–800	0.500 in.	No. 7-54	None	No. 3-73	10	457[b]	IP-21
Visual–UV	258 (UV)	200–375	0.625 in.	None	0.125 in.	No. 7-54	10	258[c]	IP-28
	410 (visual)	375–800	0.250 in.	None	None	None	10	410[c]	IP-28

[a] Farrand analyzer. [b] With emission monochromator in position. [c] With emission monochromator removed and 1P-28 photomultiplier in its place.

A calibration curve of peak area (square centimeters) *versus* concentration (nanograms) of each acridanone derivative obtained from the respective added authentic standards of I, II, and III (Fig. 2A) is constructed, from which the concentration of these compounds in the unknowns can be determined by interpolation.

The overall recovery of the added authentic standards of I, II, and III is determined by comparison of the fluorescence emission (as peak area) of their respective recovered 9-acridanones against that of the respective authentic standards scanned on the chromatoplate. Percent recovery should be determined routinely as a check on the reproducibility of the assay.

Analysis of Benzophenones in Urine by Spectrophotodensitometry—The major urinary metabolite (II), which accounts for 40–55% of a given dose, is present as a glucuronide conjugate along with minor amounts of I, III, IV, and V (Scheme I). To 1.0 ml of urine, add 5 ml of 6 *N* HCl, mix well, and extract with 2 × 10 ml of ether to remove interfering substances. The aqueous phase is hydrolyzed, and the benzophenones formed are extracted from the hydrolysate as previously described (17) and analyzed by TLC but with the following modifications.

Dissolve the ether residues containing the basic benzophenones (extracts C and D) (Scheme II in Ref. 17) in 100 μl of acetone, transfer a 25-μl aliquot of the extract onto a 20 × 20-cm silica gel G chromatoplate[5] as before, and develop as described (17). The concentration of each component in the unknowns is determined by comparison to 5.0 or 10.0 μg of their respective authentic standards recovered from urine as previously described (17).

The sensitivity of the instrument for the benzophenones in the visual–UV mode is 0.5–1.0 μg measured at 258 nm. The major urinary metabolite (II) is usually present in sufficient concentration to be readily quantitated as its benzophenone (VII) (R_f 0.38), whereas the amounts of VI (R_f 0.23) and VIII (R_f 0.62) (Fig. 1B) present may be at the sensitivity limit of the instrument and not accurately measurable. In this event, the benzophenones can be eluted separately into methanol, cyclized to the 9-acridanone derivatives, and quantitated fluorometrically as described (17).

Table II—Reproducibility (Mean and Standard Deviation) of the Measurement of Varying Concentrations of the 9-Acridanones on the Same Chromatoplate

Concentration Applied to Plate, ng	Instrument Gain Setting	Peak Area, cm^2 [a]		
		X	XI	XII
1	10	0.71 ± 0.06	0.87 ± 0.07	2.34 ± 0.16
10	3	1.86 ± 0.13	2.75 ± 0.09	4.00 ± 0.18
25	1.5	2.52 ± 0.24	3.75 ± 0.05	5.00 ± 0.24
50	1.5	5.18 ± 0.33	6.64 ± 0.16	9.05 ± 0.25
100	0.75	4.68 ± 0.07	6.63 ± 0.08	7.50 ± 0.14

[a] Mean of three individual measurements.

RESULTS AND DISCUSSION

Method Conditions—The spectrophotodensitometer[2] used can be operated only in the reflectance mode (20) and is claimed to have certain advantages over measurement in the transmission mode, which is possible with other commercial instruments[6]. The most significant advantages of spectrofluorodensitometry in the reflectance mode are the minimization of energy losses due to internal absorption or self-quenching due to the thickness of the silica gel bed and transmission losses through a glass plate.

Commercially available TLC plates of 250-μm bed thickness and 60-μm particle-size silica gel G[5] gave reproducible data with respect to reproducibility of peak area measurement of varying concentrations of the 9-acridanones on the same chromatoplate (Table II) and between chromatoplates (Table III). The reproducibility data of these fluorometric measurements using the reflectance mode are in acceptable agreement with data reported using

[5] Chromatoplates were manufactured by E. Merck (F_{254}), Darmstadt, Germany, and marketed by Brinkmann, Westbury, N.Y.

[6] *E.g.*, Zeiss (2, 3) and Schoeffel (4, 6, 12, 13).

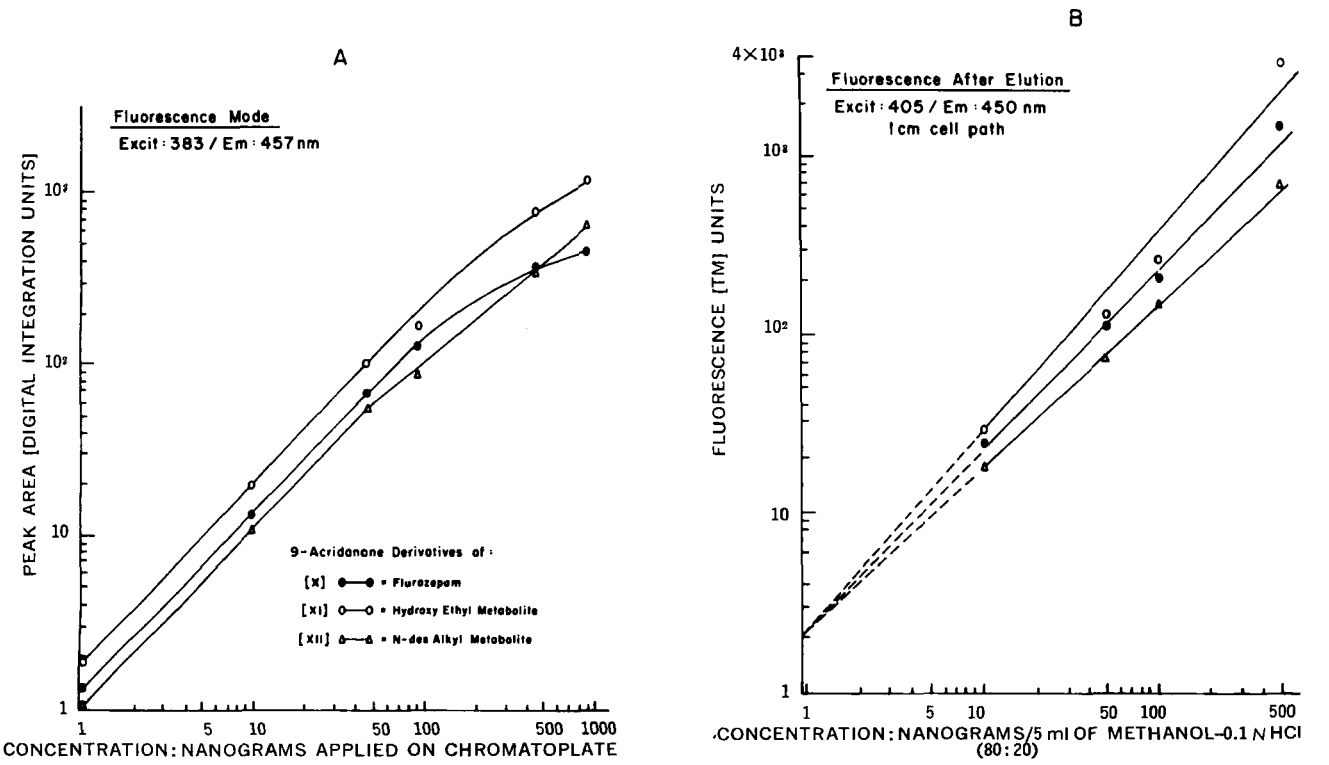


Figure 2—*Calibration curves of the 9-acridanones determined by: (A) spectrofluorodensitometry, and (B) solution fluorometry after elution.*

other equipment in the same mode and similar chromatoplates (2–6, 12, 13, 21, 22). It is essential to run at least one set of standards on each chromatoplate for purposes of calibration and quantitation of unknowns run on the same plate.

Blood levels of flurazepam following 30-mg therapeutic doses are barely detectable (23) by solution fluorescence (after elution); however, they can be quantitated with much greater precision by direct scanning fluorometry. The signal-to-noise ratio at the gain setting used to measure these levels is sufficient that further amplification of the signal can be employed without significantly increasing the background. Consequently, flurazepam levels as low as 0.1–0.2 ng/ml of blood (using a 4-ml specimen/assay) may be detected by direct scanning. This is feasible mainly due to the high intrinsic fluorescence of the 9-acridanones and the absence of spurious fluorescent impurities from the biological extracts that may interfere with the measurement.

The acceptable agreement seen in the blood levels of each component measured by both methods (Fig. 3) is especially encouraging with respect to the saving in analysis time, realized by eliminating the elution step, and the higher sensitivity (almost a fourfold increase) and precision attainable using the scanner. Although solution fluorescence measurements have a much wider linear dynamic range of concentration than direct scanning (Fig. 2), the latter enjoys certain distinct advantages with respect to minimization of solvent interaction and quenching effects, concentration of the compound to be measured in a small well-defined band, and a superior signal-to-noise ratio which can be further amplified to increase sensitivity. These factors offset the disadvantages of a limited linear dynamic range and the need to run reference standards for calibration of each chromatoplate to compensate for any variability of fluorescence emission between plates (4, 12, 13, 21).

Table III—Reproducibility (Mean and Standard Deviation) of the Measurement of Varying Concentrations of the 9-Acridanones Measured on Different Chromatoplates

Concentration Applied to Plate, ng	Peak Area, cm²[a]		
	X	XI	XII
1	0.24 ± 0.01	0.29 ± 0.02	0.30 ± 0.04
10	2.40 ± 0.05	3.35 ± 0.13	3.20 ± 0.17
25	5.58 ± 0.26	8.43 ± 0.26	7.63 ± 0.06
50	11.40 ± 0.34	16.03 ± 0.75	14.87 ± 0.15
100	21.70 ± 1.68	30.70 ± 0.86	27.75 ± 1.85

[a] Mean of individual peak area measurements [corrected to the same instrument gain factor (3.0) made on three separate plates].

The analysis of urinary metabolites by scanning in either the fluorescence or visual-UV mode is hindered by significant amounts of endogenous UV-absorbing or fluorescent components which interfere with their quantitation. Although washing the acidified urine with ether prior to hydrolysis removes significant amounts of UV-absorbing endogenous phenolic and indolic acids, sufficient amounts of UV-absorbing and fluorescent components are still extracted into ether after hydrolysis from the alkalinized urine. The use of an aliquot of the total extract for TLC separation reduces the amount of interfering substances but at the expense of sensitivity.

The benzophenones are intensely yellow in color and can also be quantitated in solution at 415 nm after elution into methanol (17). The low energy output of the xenon continuum at 415 nm reduces the sensitivity limits of detection, thereby nullifying the potential utility of direct scanning. It was evident that extensive sample clean-up was required prior to TLC analysis to enable precise and sensitive quantitation at 258 nm by direct scanning. Due to these limitations and also to the fact that the benzophenone of metabolite II was the major measurable component present, it was more convenient to elute this component and quantitate it in solution as previously described (17).

Application of Method to Clinical Specimens—Two subjects (C.R. and N.S.), free of previous benzodiazepine therapy, were selected and administered a single 30-mg dose of flurazepam[1] orally. Blood specimens were drawn prior to medication (control) and at 1, 3, 6, 12, and 24 hr postmedication. The fluorescence of the 9-acridanone derivatives separated by TLC analysis was first determined using the direct scanning TLC analyzer and then redetermined in solution after the compounds were eluted from the silica gel into methanol–0.1 *N* HCl (80:20) as described (17).

The blood level data obtained by the two procedures for flurazepam and its major blood metabolites (II and III) in these two subjects are shown in Fig. 3. Measurable levels of all three components were seen in both subjects. The amount of flurazepam in

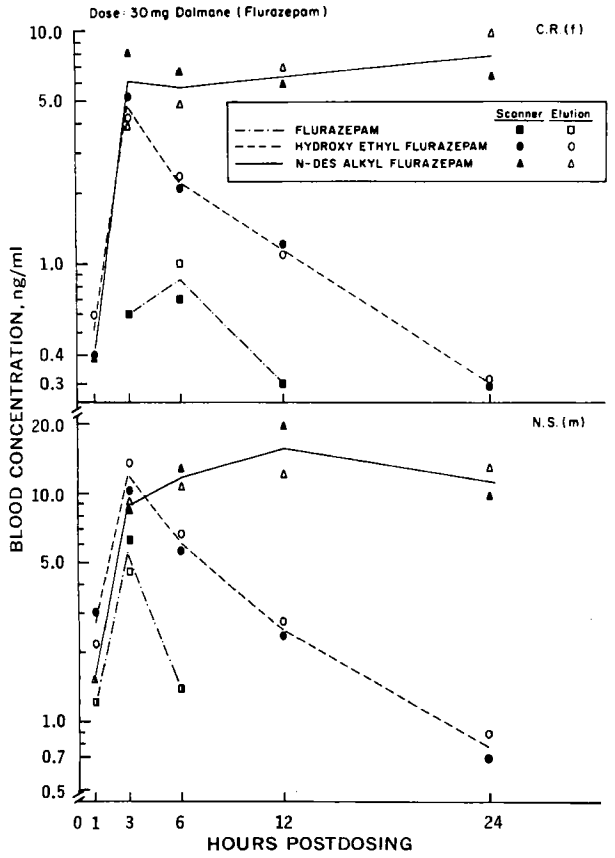


Figure 3—*Blood levels of flurazepam and its major blood metabolites in humans determined by fluorometry after elution* versus *scanning fluorodensitometry.*

each subject was very low, being at about the limit of detectability by elution, although measurable with adequate sensitivity by scanning densitometry. In Subject C.R., trace flurazepam levels of 1 ng or less/ml of blood were seen between 3 and 12 hr; in Subject N.S., a maximum flurazepam level (mean = 5 ng/ml) was seen at 3 hr and declined to nonmeasurable amounts by 12 hr.

The amounts of metabolites II and III, however, were readily measurable by both methods 1 hr after dosing. In both patients the hydroxyethyl metabolite (II) showed maximum levels at 3 hr, declined thereafter, and were measurable up to 24 hr. The concentration of *N*-desalkyl flurazepam (III), the major metabolite, increased with time, reaching a mean concentration of 10–12 ng/ml at 24 hr.

In another study (23) in four subjects following chronic administration of a single 30-mg oral dose daily for 14 consecutive days, the *N*-desalkyl metabolite (III) was the major metabolite in blood, reached steady-state levels after 7 days, and was eliminated with an "apparent" half-life ranging from 47 to 100 hr.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 11, 1974, from the *Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche, Inc., Nutley, NJ 07110*

Accepted for publication June 19, 1974.

The authors thank Dr. R. Pocelinko for conducting the clinical study at the Beth Israel Hospital, Newark, N.J., Mr. S. Cravitt, Senior Staff Engineer, Farrand Optical Co., for his generous assistance, and Mr. R. McGlynn for the drawings of the figures presented.

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