

Blood Level Distribution Patterns of Diazepam and Its Major Metabolite in Man

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The effect of different diazepam dosage regimens on the blood level patterns of diazepam (I) and of its metabolite *N*-desmethyl diazepam (II) in man was studied, using specific GLC and TLC techniques, and an ultraviolet assay measuring both components. Single oral doses of diazepam (10 mg.) produced low (0.18–0.21 mcg./ml.) and rapidly declining diazepam (I) blood levels. Repeated daily doses (30 mg.) caused a progressive increase of diazepam (I) levels. The metabolite (II) appeared 24–36 hr. after the first dose, and, thereafter, the levels increased rapidly, approaching those of I. Upon discontinuing the drug after repeated dosing, components I and II disappeared from blood very slowly, II persisting longer than I. The patterns indicated a redistribution into blood of I and II previously stored by tissues. Following chronic massive doses (150–200 mg./day), levels of I averaged 1.60 mcg./ml. (after 1 week of continuous dosage), while II continued to increase until an apparent equilibrium was reached at a ratio of I–II of 1:2.5. Only traces of the urinary metabolites, oxazepam and 3 OH-diazepam, were identified in blood.

DIAZEPAM,¹ 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one (Ro-S-2807) is a psychotherapeutic agent, synthesized by Sternbach and Reeder (1), which among the other members of the benzodiazepine class of compounds is distinguished by tranquilizing and antitension activity combined with marked muscle tone regulating properties (2). The correlations between pharmacological activity and chemical structure of the 1,4-benzodiazepine derivatives have been reviewed by Childress and Gluckmann (3).

Distribution and fate of diazepam labeled with tritium in the C₆-phenyl ring have been studied by Schwartz *et al.* (4). Following the oral administration of 10 mg. of diazepam, blood level maxima of about 0.10 mcg./ml. were found after 1–2 hr. Blood level fall-off patterns indicated a rapid and extensive uptake by tissues. Although the radioactivity in the blood appeared to represent mainly the intact drug, diazepam was shown to be excreted exclusively in the form of its metabolites. The major metabolic pathways were shown to consist of demethylation at the nitrogen in position 1, addition of a hydroxyl group at carbon 3, and conjugation of the respective derivatives (Scheme II). Oxazepam glucuronide was the predominant metabolite in urine. *N*-Demethylated diazepam was also found in urine, and in addition could be detected in the blood several hours after a 10-mg. single diazepam

dose. The excretion rates of the radioactivity indicated an over-all drug half-life of 2–3 days.

Blood level studies on larger groups of patients under different diazepam dosage conditions employing a U.V. spectrophotometric analytical procedure yielded inconclusive results on account of the limited sensitivity and specificity of that method. Subsequently, a gas liquid chromatographic (GLC) assay was developed capable of measuring nanogram quantities (10⁻⁹ Gm.) of diazepam with a high degree of precision and selectivity (5). This procedure established its usefulness in investigations of the placental transfer of diazepam (6), and also permitted the determination of the major metabolite in blood from diazepam treated patients in a single assay.

The objective of the present study was to establish blood level distribution patterns of diazepam and its metabolite produced under a variety of diazepam dosage conditions, by means of carefully designed and controlled clinical studies, and by very sensitive and specific analytical methodology. Blood level curves are reported for single therapeutic doses, administered by oral, intravenous, and intramuscular routes, for repeated oral therapeutic doses and for chronic treatment with massive doses. Three independent analytical procedures and improved modifications thereof are described and their scopes are compared, including (a) a quantitative spectrophotometric assay of more limited sensitivity and specificity but of usefulness in toxicology; (b) a modified quantitative gas liquid chromatographic procedure differentiating between the intact drug and its *N*-demethylated metabolite; and (c) a qualitative thin-layer chromatographic (TLC) technique capable of definite identification of the individual benzodia-

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¹ Marketed as Valium by Hoffman-La Roche, Nutley, N. J.

zepin derivatives in extracts of biological materials.

ANALYTICAL PROCEDURE

U.V. Assay.—The U.V. method measures both intact diazepam and its major metabolite, *N*-demethylated diazepam (Ro 5-2180). Both of these compounds are extractable into diethyl ether from blood at pH 7.0 and exhibit similar U.V. absorption spectra with maxima at 240 and 285 $m\mu$, respectively (Fig. 1), the absorbance at 240 $m\mu$ being twice that at 285 $m\mu$.

Two-milliliter aliquots of oxalated specimens of control blood taken from the patient prior to medication and a 2-ml. specimen of control blood containing 5.0 mcg. of standard diazepam from a stock solution were run along with the unknowns. The samples were extracted twice with ether by a procedure described previously (5), and the extracts were combined. The combined ether extracts were washed twice with 5 ml. of 0.1 *N* NaOH, centrifuged, and the NaOH layer removed with a capillary pipet. The ether was extracted with 2 ml. of 2 *N* HCl which was then washed twice with 10-ml. portions of ether, centrifuged, the ether removed by aspiration. The ether washed HCl extract was transferred into a micro quartz cell (0.6-ml. capacity) with a clean glass capillary pipet. Using a 1-mm. pinhole slit in a Beckman DU spectrophotometer in conjunction with the micro quartz cell, the U.V. absorption spectrum of control, internal standard, and sample blood extracts were scanned by measuring their respective absorbances at 230, 240, 250, 265, 275, 285, 295, and 320 $m\mu$ to establish the presence of the characteristic diazepam spectrum with maxima at 240 and 285 $m\mu$.

The absorbances (*A*) of the internal standards and the unknowns at 240 and 285 $m\mu$ were corrected for the corresponding control absorbance. The concentration of the unknowns was calculated from the corrected absorbance values at 240 $m\mu$ of the internal standard. The recovery of 5.0-mcg. amounts of diazepam and Ro 5-2180 added to blood was $90 \pm 5.0\%$ and was determined from the absorbance at

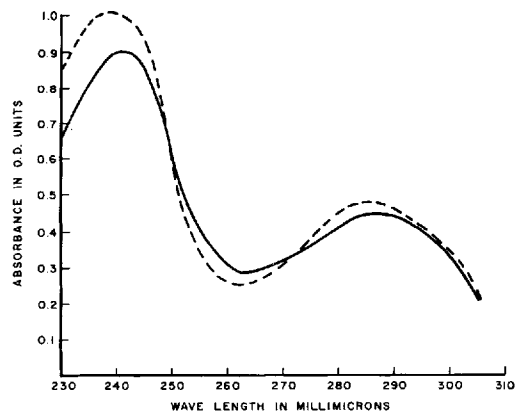


Fig. 1.—U. V. absorption spectra of diazepam and its metabolite (Ro 5-2180) in 2 *N* HCl (10 mcg./ml.). Key: —, diazepam $A_{240} = 0.091/\text{mcg.}$; -----, metabolite $A_{240} = 0.101/\text{mcg.}$

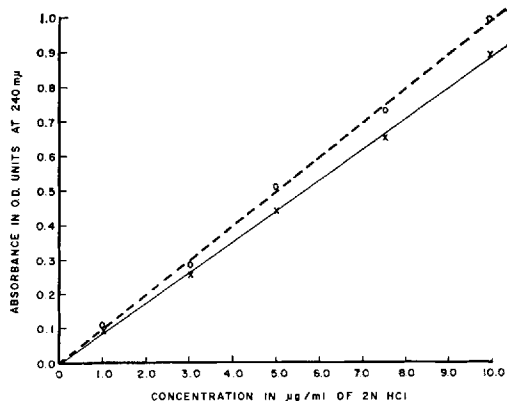


Fig. 2.—Standard curves of diazepam and its metabolite (Ro 5-2180) at 240 $m\mu$. Key: \times , diazepam; \circ , Ro 5-2180.

240 $m\mu$ in a 1-cm. cell of standard solutions of diazepam and Ro 5-2180, respectively. The A_{240} for 1 mcg./ml. solutions was 0.091 and 0.101, respectively. The method has a sensitivity limit of 0.3–0.5 mcg./ml. of blood, and a range of linearity up to 10 mcg./ml. of final solution (Fig. 2) and is sufficiently sensitive to measure blood levels resulting from doses greater than 50 mg.

Separation of Diazepam and Its Metabolites by Thin-Layer Chromatography.—Thin-layer chromatography was employed for the qualitative identification of the presence of diazepam and its metabolites extracted from blood and assayed cumulatively in the 2 *N* HCl acid extract by the U.V. method. Following spectrophotometric measurement, the acid extract was neutralized with 2 *N* NaOH to a blue end point, using bromothymol blue indicator, and extracted into diethyl ether which quantitatively removes diazepam and the metabolites. The residue of this extract was dissolved in 0.2 ml. of *n*-hexane (Fisher spectrograde) and quantitatively transferred onto a thin-layer plate of Silica Gel G (Stahl) containing a fluorescent indicator. Pure standards of diazepam and other possible metabolites were run alongside the sample extracts for identification of the compounds.

The plates were developed for about 2 hr. in chloroform–acetone, 90:10, and then viewed under ultraviolet shortwave light to identify the compounds on the plate. A typical chromatogram is shown in Fig. 3. The results were further verified by two-dimensional thin-layer chromatography using either chloroform–heptane–ethanol (10:10:1) followed by chloroform–heptane–acetic acid–ethanol (5:5:1:0.3) or chloroform–acetone (90:10) followed by chloroform–heptane–ethanol (10:10:1) as the developing solvents. A typical chromatogram is shown in Fig. 4.

Determination of Diazepam and Its Metabolites by Gas-Liquid Chromatography.—The more sensitive and specific gas chromatographic method is capable of quantitatively resolving diazepam and its major metabolite (Ro 5-2180) in blood, after they are hydrolyzed to their respective benzophenones (5) (Scheme I). By this method the amount of diazepam and Ro 5-2180 can be determined in the same sample of blood in a single assay.

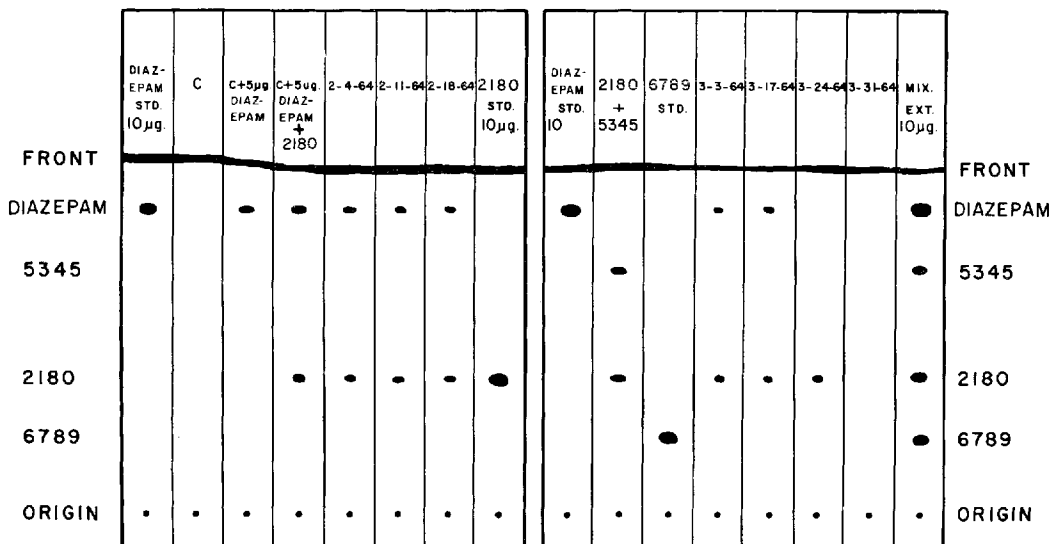


Fig. 3.—Thin-layer chromatograms of blood extracts showing the presence of intact diazepam and its *N*-demethylated metabolite, Ro 5-2180, in a patient maintained on high doses of diazepam for an extended period of time. Silica Gel G (Stahl) with fluorescent indicator. Solvent: chloroform-acetone, 90:10 v/v, 2 hr.

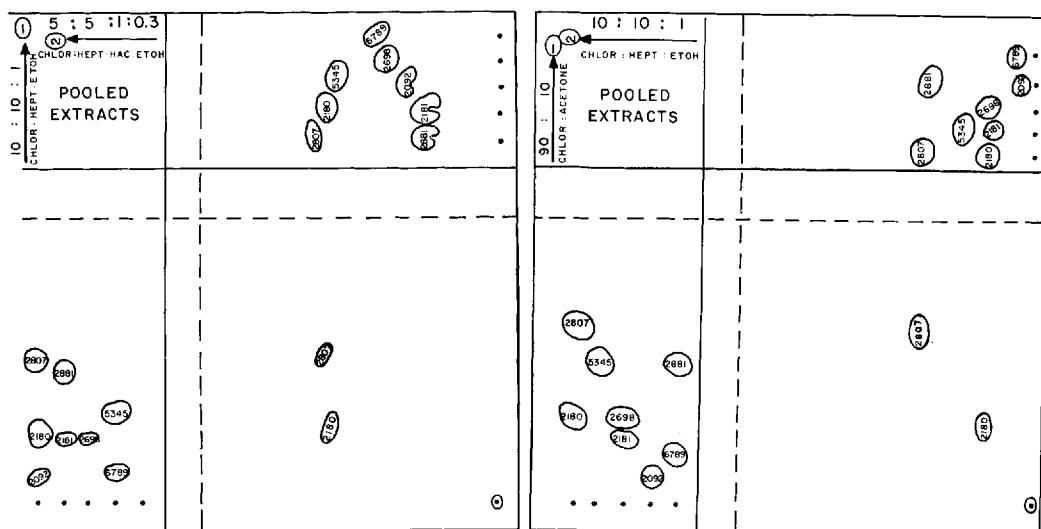
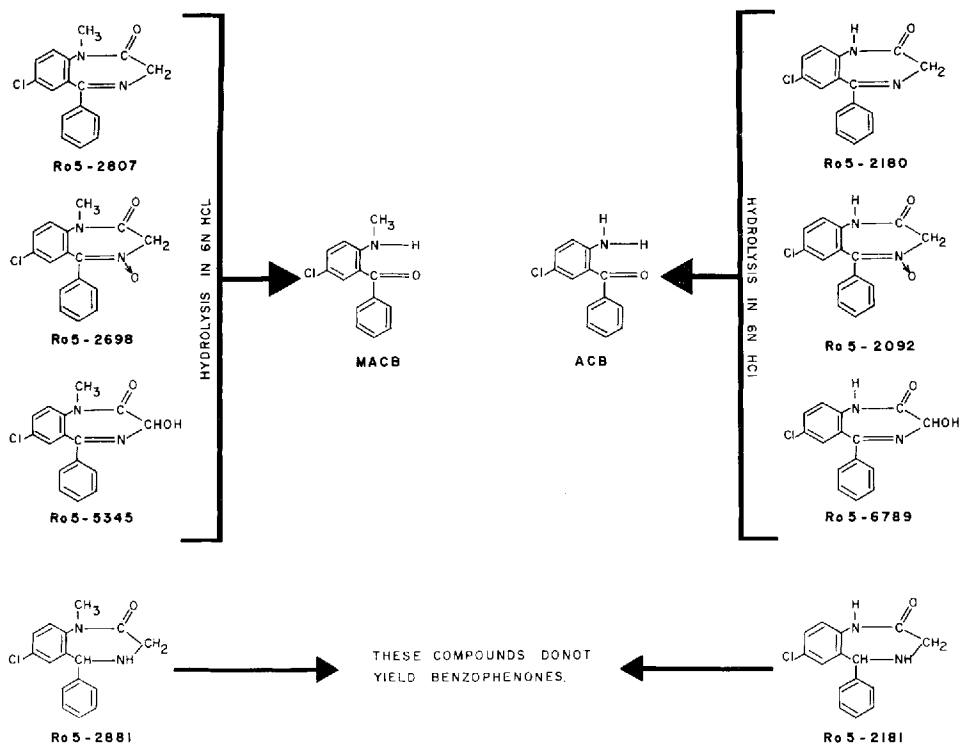


Fig. 4.—Two-dimensional thin-layer chromatograms of pooled blood extracts showing the presence of intact diazepam (Ro 5-2807) and its major metabolite (Ro 5-2180).

An improved procedure is described employing a liquid phase of 2% Carbowax 20M-terephthalic acid (CBW-20M-TPA), a synthetic polyester (7), which was found to be superior to Carbowax 20M used in the original method. It is a more polar phase with greater temperature stability, bleed resistance, column life, uniform coating characteristics, and produces very sharp well-resolved symmetrical (Gaussian shaped) peaks (Fig. 5). Its physical characteristics make it very selective for a number of benzophenones. The sample preparation for gas chromatography was carried out exactly as published (5).

Gas chromatographic parameters were obtained

on a Jarrell-Ash instrument model Universal 26-700 equipped with an electron capture detector (No. 26-755) containing a 100 mc. titanium tritide β ionization source. Column: a 2-ft. column of 2% Carbowax 20M-terephthalic acid polyester phase on silanized Gas-Chrom P 100/120 mesh contained in $\frac{1}{4}$ in. stainless steel tubing was used. Carrier gas: nitrogen (oil pumped and dry) passed through a molecular sieve before entering the column was adjusted to a flow rate of 150-170 ml./min. measured at room temperature. The column head pressure was 17-20 psig on the second stage of the gas regulator. Condition of column head pressure and flow rate may be varied to obtain a retention time



Chemical structures and reactions of diazepam (Ro 5-2807) and some of its analogs. MACB = 2-methyl-amino-5-chloro-benzophenone. ACB = 2-amino-5-chloro-benzophenone.

Scheme I

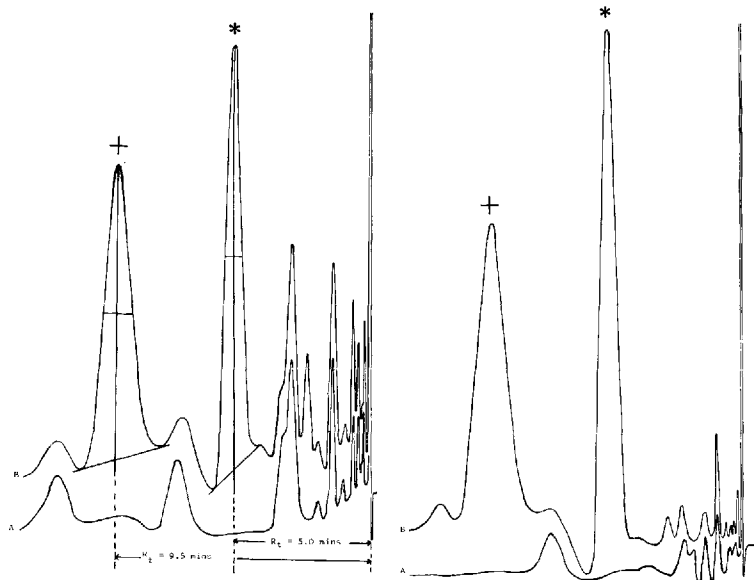


Fig. 5.—Chromatogram of diazepam and its *N*-demethylated metabolite determined by GLC as the MACB and ACB derivatives from blood extracts of patient L. R.; 30 mg. of diazepam per day for 10 days. Key: *, MACB; +, ACB; A, control blood from patient; B, patient blood after medication. Left, GLC assay of blood ether extract (10/100 μ l.). Right, two-dimensional TLC of pooled blood ether extract and GLC assay of the two components (2/300 μ l.).

(R_t) of 5–6 min. for MACB (diazepam) and 9–10 min. for ACB (Ro 5-2180) for effective resolution from adjacent peaks.

Temperatures: injection port, $250^\circ \pm 2.0$; detector, $210^\circ \pm 2.0$; oven, $215^\circ \pm 2.0$ (isothermal); amplifier range, 10×10^{-9} amp. full-scale deflection (fsd); recorder, Bristol, output, 10 mv.; time constant, 1 sec. (fsd); chart speed, 1.25 cm./min. = 30 in./hr.; detector voltage, 20–30 v.

d.c. Optimal detector voltage has to be determined for each compound assayed and should be checked frequently to compensate for any changes in detector response due to variation in standing current. Minimum detectable amounts of MACB = 5.0×10^{-9} Gm. (5 nanograms) and ACB = 10×10^{-9} Gm. (10 nanograms).

Preparation of Column Substrate.—The inert support Gas-Chrom P 100/120 mesh (Applied Sci-

ence Laboratories, State College, Pa.) was silanized according to the method described by Horning *et al.* (1959) to inactivate any adsorbent sites present. Two grams of Carbowax 20M-terephthalic acid (TPA) polyester phase (Wilkins Instrument and Research, Inc., Walnut Creek, Calif.) dissolved in 500-ml. of hot methylene chloride and 98 Gm. of silanized Gas-Chrom P 100/120 mesh were shaken for 30 min. on a reciprocating shaker with intermittent release of pressure. The material was transferred into a flat dish and the solvent evaporated off on a hot plate with continuous stirring. The coated substrate was then dried overnight in an oven at 200°, cooled to room temperature, and stored in an airtight container. A 2-ft. piece of stainless steel or aluminum tubing was packed with the prepared substrate and conditioned for 48-72 hr. at 230-240°. The column was then ready for use, and had a useful life span of at least 6 months of continuous use.

Preparation of Standard Curves of MACB and ACB.—2-Methylamino-5-chlorobenzophenone (MACB) and 2-amino-5-chlorobenzophenone (ACB) synthesized by Sternbach *et al.* (8), of at least 99% purity, were dissolved in *n*-hexane (Fisher spectrograde) to yield separate stock solutions of 1 mg./ml. From this solution suitable dilutions were made in *n*-hexane to yield final solutions covering the range of 5 ng./10 μ l. to 30 ng./10 μ l. Three 10- μ l. aliquots of each of the final solutions were injected into the gas chromatograph and from their average peak area a standard curve of peak area (cm.²) versus nanograms of MACB or ACB was drawn. A standard curve should be determined for each day of analysis because column performance and detector response to these compounds changes with time.

Preparation of Standard Solutions of Diazepam and Ro 5-2180.—Diazepam and Ro 5-2180, synthesized by Sternbach and Reeder (1), of pharmaceutical grade purity (>99%), was used in the preparation of pure standard solutions.

Ten milligrams of each pure compound was weighed into separate 100-ml. volumetric flasks, and dissolved in 10-15 ml. absolute ethanol, warming the flask if necessary to effect solution. The solutions were made to volume with absolute ethanol, and should be water white in color.

The stock solution (A) contains 100 mcg./ml. One milliliter of solution A was transferred into another 100-ml. volumetric and diluted to volume with distilled water. The standard solution (B) contains 1 mcg./ml.

Suitable aliquots of solution B were used for obtaining recovery data from blood. Solutions A and B should be made fresh daily.

RESULTS AND DISCUSSION

The blood level distribution patterns of diazepam and its *N*-demethylated analog (Ro 5-2180) in man were determined by a gas-chromatographic procedure (5), which has an over-all recovery of 90% \pm 6.0 and a sensitivity limit of 0.02-0.03 mcg. of diazepam/ml. of blood. The recovery of Ro 5-2180 is of the order of 70% \pm 7.0 with a sensitivity limit of 0.05-0.10 mcg./ml. (Table I.)

Diazepam Blood Levels Following Single Doses.—Blood level fall-off curves following single 10-mg. oral doses (Fig. 6), indicated that peak blood diazepam levels ranging from 0.18-0.22 mcg. were obtained 1 hr. after dosing, after which these levels

TABLE I.—RECOVERY OF DIAZEPAM AND RO 5-2180 ADDED TO 1 ml. OF BLOOD, DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

Diazepam Added, ng.	Recovery of Diazepam		% Recovery
	Total MACB Recovered, ng.	Diazepam Recovered, ng. ^a	
100.0	78.0	90.5	91.0
100.0	75.0	87.0	87.0
200.0	147.0	171.0	86.0
200.0	152.0	176.0	88.0
200.0	144.0	167.0	84.0
200.0	148.0	172.0	86.0
200.0	164.0	190.0	95.0
200.0	172.0	199.0	99.8
200.0	162.0	188.0	94.0
200.0	160.0	186.0	93.0
200.0	143.0	165.0	83.0
200.0	143.0	165.0	83.0
200.0	155.0	179.0	85.0
200.0	159.0	184.0	92.0
200.0	164.0	190.0	95.0
200.0	163.0	189.0	94.0
200.0	162.0	188.0	94.0
200.0	167.0	194.0	97.0
200.0	170.0	198.0	99.0
200.0	166.0	193.0	97.0
300.0	225.0	261.0	87.0
300.0	218.0	253.0	84.0
300.0	210.0	244.0	81.0
300.0	227.0	263.0	88.0
300.0	205.0	238.0	80.0
		Over-all av.:	90.0% \pm 6.0
Ro 5-2180 Added, ng.	Recovery of Ro 5-2180		% Recovery
	Total ACB Recovered, ng.	Ro 5-2180 ^b Recovered, ng.	
400.0	265.0	307.0	77.0
400.0	260.0	302.0	76.0
400.0	225.0	261.0	65.0
400.0	228.0	265.0	66.0
600.0	436.0	506.0	84.0
600.0	434.0	503.0	84.0
600.0	355.0	412.0	69.0
600.0	338.0	392.0	65.0
600.0	320.0	371.0	62.0
600.0	344.0	399.0	67.0
600.0	356.0	413.0	69.0
600.0	360.0	418.0	70.0
600.0	344.0	399.0	67.0
600.0	338.0	392.0	65.0
		Over-all av.:	70.5% \pm 7.0

^a ng. MACB \times 1.16 = ng. diazepam. ^b ng. ACB \times 1.16 = ng. Ro 5-2180.

declined rapidly within 6 hr. (half-life = 2-3 hr.) to a plateau level of 0.04-0.05 mcg./ml. This level was maintained for 12-24 hr. after which a gradual decline was seen (half-life = 27-28 hr.). These data appear to confirm the fall-off pattern using ³H-labeled diazepam where the ether extractable radioactivity was measured by liquid scintillation counting.

In another study using a parenteral formulation (F-126) a group of three patients received a single 10-mg. intravenous dose of diazepam, while another group of three patients received the same formulation as a single 10-mg. intramuscular dose. The blood level curves (Fig. 7) indicate that the blood level maxima obtained with the parenteral formulation were of the same order of magnitude as those obtained with the oral doses. As before, the maxima were followed by a decline in the blood level

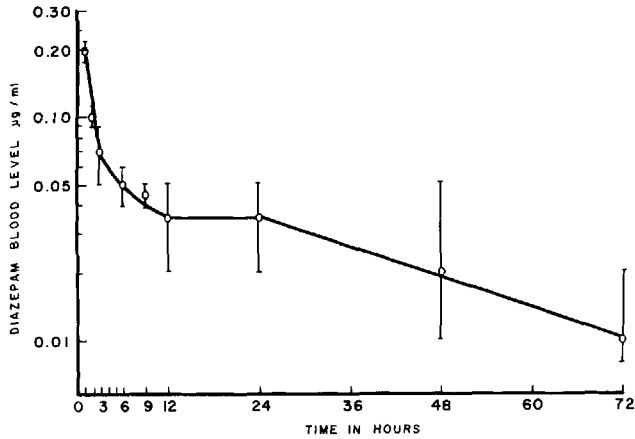


Fig. 6.—Blood level fall-off curve in man following a single oral 10-mg. dose of diazepam.

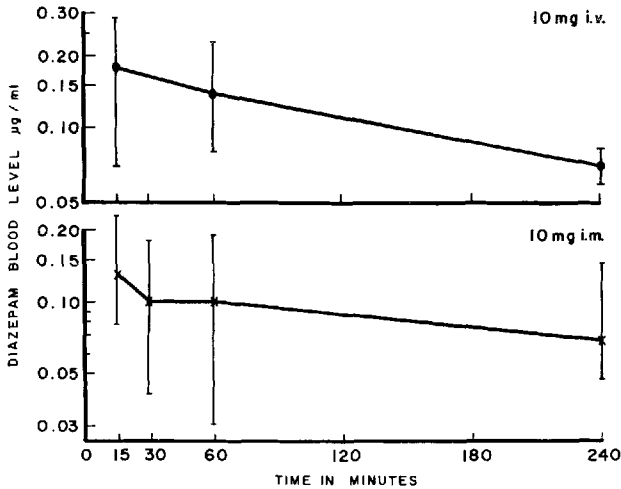


Fig. 7.—Blood level fall-off curves in man following a single 10-mg. dose of diazepam in a parenteral formulation by i. v. and i. m. routes.

within 4 hr. (half-life = 2.3 hr., indicating removal into tissue storage depots. No measurable amounts of the *N*-demethylated metabolite of diazepam were seen after these 10-mg. single doses.

Blood Level Distribution Pattern of Diazepam and Its Metabolite During Administration of Repeated Doses.—In this series of experiments the blood level patterns of diazepam and its *N*-demethylated metabolite were studied in two patients during periods of repeated daily dosage and immediately following the discontinuation of drug treatment.

Total daily oral doses of 30 mg. of diazepam (lying within the normal therapeutic range) were administered in divided doses either in the form of regular 10-mg. tablets (t.i.d.) or in the form of 15-mg. (7.5 + 7.5 mg.) repeat action tablets (b.i.d.). Blood specimens were drawn for analysis every 12 hr. (8 a.m. and 8 p.m.) during the entire experimental period; and, in addition at 1, 4, and 6 hr. after the first dose.

One patient (L. R.) received the drug for 10 consecutive days which represented a cumulative dose of 300 mg. He was taken off medication for 7 days, after which diazepam treatment was resumed for 10 more days, giving a second cumulative dose of 300 mg. The other subject (H. W.) was treated for 5 days with a cumulative dose of 150 mg. After a drug free period of 7 days, he received diazepam treatment for another 5 days or a second 150-mg.

cumulative dose. The two dosage forms were alternated and their sequence was reversed in a crossover fashion in the two subjects. This was of no consequence to the particular purpose of this study.

All the specimens were assayed for intact diazepam and its *N*-demethylated metabolite by GLC. The blood levels of diazepam and of Ro 5-2180 are plotted against time in Figs. 8 and 9, respectively. In both patients the curves for diazepam and the metabolite are seen to follow basically very similar patterns. Diazepam blood levels after the expected initial drop from the maxima after the first dose, increased with each day of continued medication. In the first subject (L. R.) the diazepam levels rose from a 24 hr. value of 0.18 mcg./ml. to a final value of 0.8-1.0 mcg./ml. during the 10-day period of medication. In the second subject (H. W.) there was a corresponding rise from an initial 24 hr. level of 0.12 mcg./ml. to a maximum of 0.50 mcg., within 4 days. The metabolite Ro 5-2180 could not be detected initially in either subject up to 36 hr. after the first dose, but from then on its blood levels showed a steady increase, gradually approaching the concentrations of the parent compound. This pattern was more systematic in patient L. R., especially during the first 10-day period of medication.

In both experiments discontinuation of the drug did not show an immediate change in the blood level

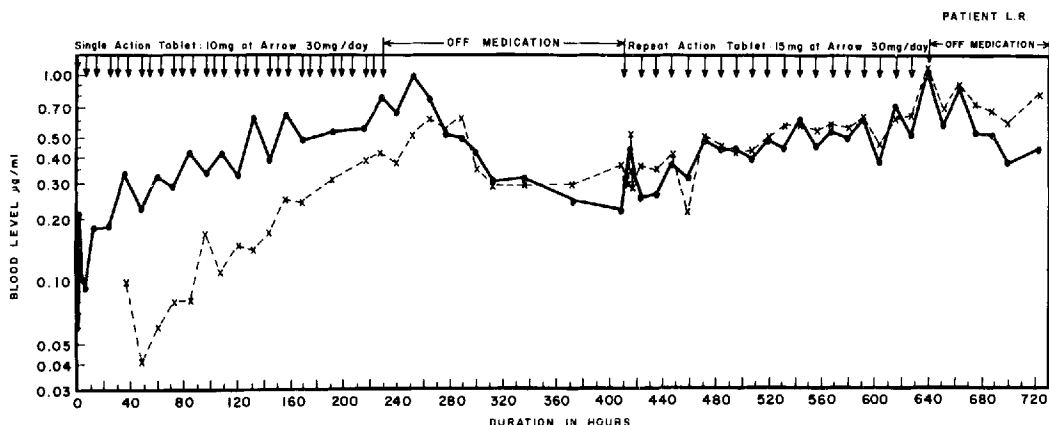


Fig. 8.—Blood level distribution pattern of diazepam and Ro 5-2180 in man following administration of single action tablets vs. repeat action tablets. Key: ●, diazepam Ro 5-2807; ×, metabolite Ro 5-2180.

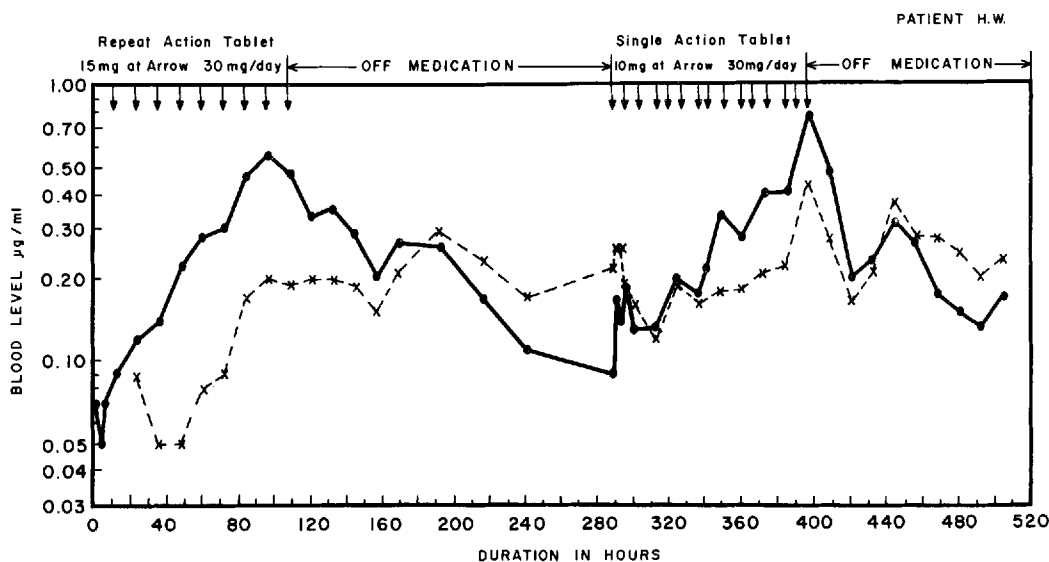


Fig. 9.—Blood level distribution pattern of diazepam and Ro 5-2180 in man following administration of repeat action tablets vs. single action tablets. Key: ●, diazepam Ro 5-2807; ×, metabolite Ro 5-2180.

of either component. The diazepam levels showed an erratic but definite decline with time but were still measurable at the end of the 7-day fall-off period. The metabolite levels fluctuated without a distinct downward trend. As a result, the ratio of the two components changed gradually in favor of the metabolite. By the end of the drug-free period the metabolite levels consistently exceeded those of intact diazepam.

Uptake of the drug during the second period of medication produced an increase in the cumulative blood levels over and above the residual threshold levels after the first fall-off period. Owing to the high residual metabolite levels, the ratio of the metabolite to diazepam was maintained close to unity, especially in patient L. R. where the earlier cumulative dose had been higher.

These blood level curves bring out the important fact that the concentration of diazepam, the ratio of diazepam to its metabolite, and also the rate of elimination of these components from blood depend not

merely on the dose administered at the time of sampling but also depend greatly on the preceding dosing history and its ultimate duration. These levels are governed specifically by the cumulative amount of drug administered continuously and by the duration of time of continuous medication. The blood level distribution pattern is influenced by all these parameters, and it is also indicative of drug accumulation in some tissue storage depots.² Release from such depots back into the blood stream is manifested by the slow and erratic saw-toothed fall-

² Tissue storage depots implies those compartments of the body which are associated in drug accumulation, metabolism, and excretion. Such depots are defined in the terminology of biopharmaceutics and pharmacokinetics as "shallow" compartments of soft extra vascular tissues, e.g., liver, kidney, intestinal tract, and other associated organs and "deep" compartments of tissues such as bone, marrow, muscle, fat, all of which are accessible and interconnected through the blood circulatory system. Consequently, chemical compounds carried in the blood can be stored and/or metabolized in these depots, and later released back into circulation prior to excretion. [See Wagner, J. G., *J. Pharm. Sci.*, **50**, 359(1961); Doluisio, J. T., and Swintosky, J. V., *Am. J. Pharm.*, **137**, 144(1965); **137**, 175(1965).]

off pattern seen after the discontinuation of dosing. The faster buildup and the much slower disappearance of the metabolite levels indicate a relatively slower metabolism and excretion of this product.

Blood Level Distribution Pattern of Diazepam and Its Metabolite During Chronic Administration of Massive Doses.—An opportunity to study the blood level distribution pattern of diazepam and its metabolites under more extreme conditions of chronic dosing was offered by a unique therapeutic

experiment. It involved a male patient (L), age 53, with a history of chronic alcoholism and related psychic disorders who was able to tolerate daily doses of diazepam as high as 200 mg. without major side effects (9).

Blood specimens were taken at weekly intervals. They were originally analyzed by the U.V. spectrophotometric assay for "total" diazepam, and then re-examined by the differential GLC procedure (Table II).

TABLE II.—BLOOD LEVELS OF DIAZEPAM AND ITS METABOLITE IN A HUMAN^a GIVEN 150–200 mg./DAY FOR 8 WEEKS

Medication, Wk.	Date Blood Drawn	Dose	Blood Level, mcg./ml.				Total Diazepam U.V. ^a	
			Diazepam	GLC 2180	Method Ratio Met./Diaz.	Diaz. Eq. of 2180		Total 1 + 2
0 (Control)	1/21/64	Diazepam Tablets	N.M. ^a	
	1/23/64	150 mg./Day began						
1	1/28/64		1.51	1.39	0.92	1.47	2.98	3.70
2	2/4/64		4.10
3	2/11/64		1.42	2.58	1.83	2.74	4.16	3.90
4	2/18/64		2.00	2.90	1.45	3.07	5.07	5.00
5	2/25/64		1.42	2.38	1.68	2.52	3.94	4.10
	2/26/64	200 mg./day began						
6	3/3/64		1.46	3.19	2.18	3.38	4.84	4.70
7	3/10/64		1.88	3.25	1.73	3.45	5.33	4.40
8	3/17/64		1.57	4.18	2.66	4.43	6.00	4.22
	3/18/64	Withdrawal began						
9	3/24/64	90 mg./day	0.65	2.49	3.83	2.64	3.29	3.98
10	3/31/64	20 mg./day	0.30	0.16	0.53	0.17	0.47	3.70
11	4/3/64	Off diazepam, on placebo	0.09	N.M.	0.09	N.M.
12	4/7/64	On placebo	N.M.	N.M.	N.M.
13	4/10/64	On placebo	N.M.	N.M.	N.M.
14	4/16/64	On placebo	N.M.	N.M.	N.M.

^a The patient was under the care of Dr. K. S. Ditman, Alcoholism Research Clinic, Department of Psychiatry, Center for Health Sciences, University of California at Los Angeles. ^b N.M. = not measurable.

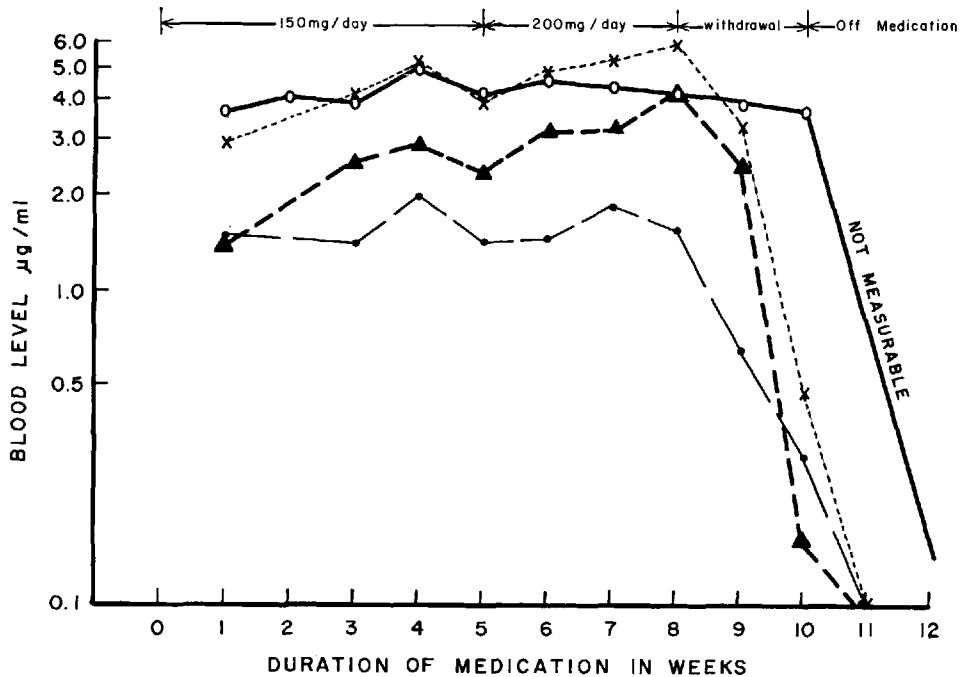


Fig. 10.—Blood levels of diazepam and its metabolite (Ro 5-2180) from a human given 150–200 mg./day orally for 8 weeks followed by gradual withdrawal of medication. Key: ●, diazepam (GLC); ▲, metabolite (2180) (GLC); ×, total diazepam equivalent GLC (calcd.); ○, total diazepam + metabolite (U. V.).

The blood level distribution pattern of the two individual components, of the calculated sum of the two, and of the total spectrophotometrically measured material are shown in Fig. 10. After a cumulative dose of 750 mg. within the first week the blood level of intact diazepam was 1.51 mcg./ml. but then tended to stabilize itself rapidly with continued medication at 1.60–1.64 mcg./ml. After 4 weeks of continuous medication at a dosage of 150 mg./day, followed by 3 weeks at an increased dosage of 200 mg./day, the blood level of intact diazepam did not show a significant increase over the first week level of this component in blood. This seemed to indicate that an equilibrium was established between drug uptake and its disposition by metabolism, excretion, and/or tissue distribution. The levels of Ro 5-2180 equaled those of the parent component by the end of the first week, but continued to rise to about twice this value during 2 more weeks of treatment. Thereafter, with fluctuation of the absolute blood levels of the two components, the ratio of the metabolite to intact diazepam maintained a fairly constant value of about 1.8, with the exception of the last week of treatment at the highest daily dose when a further increase of this ratio was noted. A more pronounced rise of this ratio during the final phase of gradual drug withdrawal was the result of a relatively faster decline of the intact diazepam blood level over that of the metabolite.

The curves for the total drug levels as measured by the two procedures indicated reasonable agreement, except for the discrepancies during the withdrawal period, which remain unexplained.

The clinical observation of an abnormally high drug tolerance suggested the possibility of either a congenital or of a drug-induced increased metabolic rate in this subject. An increased rate of diazepam metabolism due to congenital factors would manifest itself by a slower buildup of the blood levels, by a lower stabilization level, and by a more rapid fall-off after withdrawal. A drug-induced stimulation of a metabolizing enzyme system, e.g., the liver microsomal enzyme system, would be reflected in a gradual lowering of the stabilization level during continued dosing at a constant rate. The blood level curves obtained provided no conclusive evidence of such factors in operation in this patient. The diazepam blood level of 1.51 mcg./ml. produced by a cumulative dose of about 750 mg. during the initial phase appears to be in keeping with a corresponding value of 0.8–1.0 mcg./ml. produced by a 300-mg. total dose, and of a value of 0.50 mcg./ml. following a 150-mg. total dose in the earlier low dose experiments. This, therefore, indicated similar rates of drug accumulation in all three patients. The stabilization levels of both diazepam and Ro 5-2180 maintained a fairly constant value throughout the entire period of dosing at the high level. The blood level fall-off rates during the withdrawal period were difficult to evaluate in terms of possible changes in metabolic rates due to the stepwise reduction of drug discontinuation and to the insufficient number of samples measured during this period.

Since the GLC method is capable of resolving diazepam from its metabolite Ro 5-2180, the specificity of the method depends on the absence of other analogs of diazepam, which if present in blood, and/or are ether extractable, would be hydrolyzed to MACB and ACB, respectively, and give erroneous

values for diazepam and Ro 5-2180 (Scheme I). Blood specimens from the high dose patient were pooled and analyzed first by one-dimensional and then by two-dimensional thin-layer chromatography to observe the presence, if any, of other possible metabolic analogs which might interfere with the GLC assay.

The chromatoplates (Figs. 3 and 4) showed only the presence of intact diazepam and its major metabolite Ro 5-2180. This was further verified in

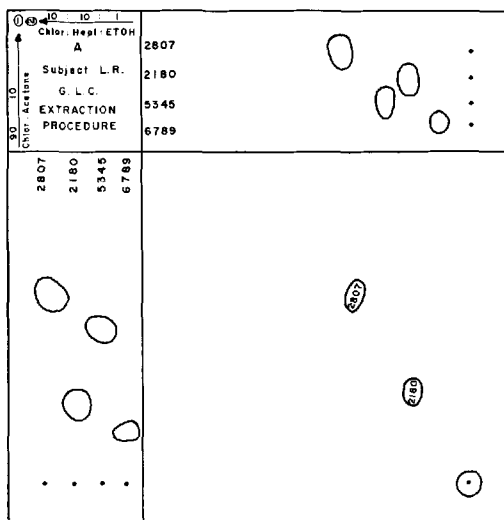


Fig. 11.—Two-dimensional thin-layer chromatogram of pooled blood extracts of a patient (L. R.) who had received 30 mg. of diazepam per day for 10 days, using the extraction procedure for GLC assay, showing the presence of only diazepam and its *N*-demethylated metabolite, Ro 5-2180.

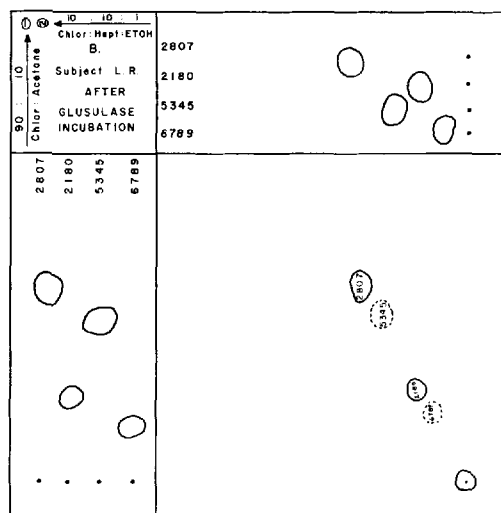
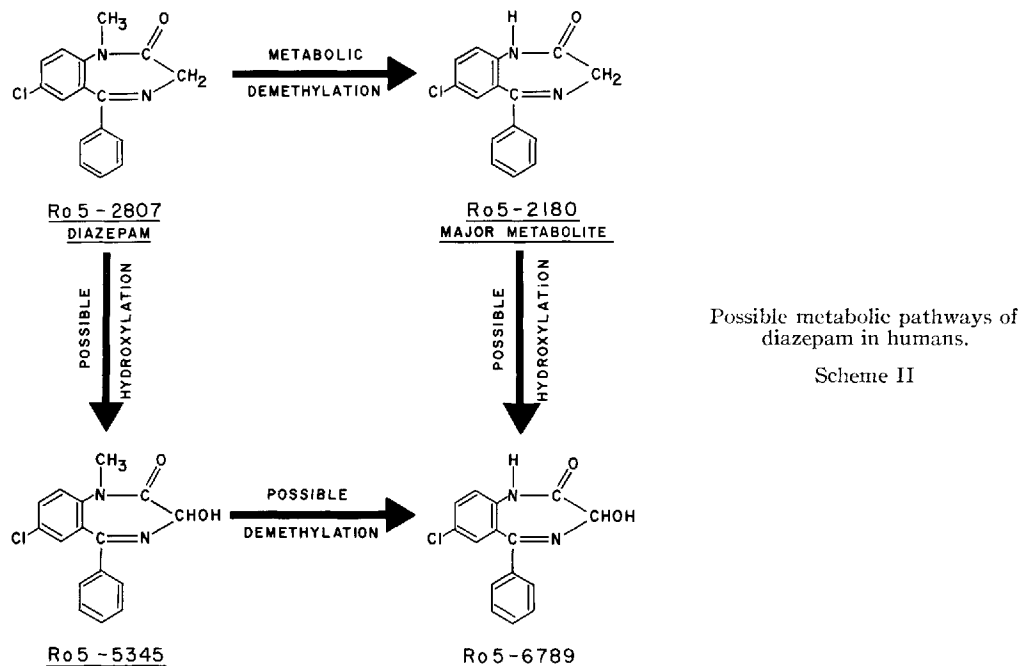


Fig. 12.—Two-dimensional thin-layer chromatogram of pooled blood extracts of a patient (L. R.) who had received 30 mg. of diazepam per day for 10 days. Using the same extraction procedure as in Fig. 11, but after incubating the blood with Glusulase enzyme, shows the presence of diazepam, Ro 5-2180, Ro 5-6789, and Ro 5-5345.



pooled blood ether extracts from the patients (L. R. and H. W.) of the low dose experiments (Fig. 11). The areas on the plate corresponding to diazepam and the metabolite were scraped off, extracted with ether, and then taken through the GLC procedure. The chromatograms (Fig. 5) establish the formation of MACB and ACB (benzophenones) from the hydrolysis of their respective parent compounds found in the blood, thus establishing the specificity of the GLC method for diazepam and Ro 5-2180. By two analytical procedures (TLC and GLC) diazepam and its *N*-demethylated analog Ro 5-2180 have been shown to be the major ether extractable components present in the blood of patients treated with the pharmaceutical formulations of diazepam.

Since the 3-hydroxy compounds, Ro 5-5345 and Ro 5-6789 (oxazepam) (10), have been shown to be significant metabolites of diazepam in human urine in the form of conjugated glucuronides (4), their presence in the blood as such was also investigated. Aliquots of blood were pooled (32 ml.), acidified with diluted HCl to pH 5.5, and incubated in a 37° water bath with 0.5 ml. of Glusulase enzyme (Endo Labs, Inc., Richmond Hill, N. Y.; activity = 100,000 units of glucuronidase, 50,000 units of sulfatase/ml.) for 2 hr., swirling every 15 min. The samples were then buffered to pH 7.0, extracted with ether, and analyzed by two-dimensional TLC using the same solvent systems as before.

The chromatoplates (Fig. 12) showed the presence of significant amounts of diazepam and Ro 5-2180 and also trace amounts of Ro 5-5345 and Ro 5-6789. After acid hydrolysis and GLC analysis, the identity of diazepam and Ro 5-2180 recovered from the chromatoplates was again established with significant amounts of their respective benzophenones, whereas Ro 5-5345 and Ro 5-6789 present in trace amounts gave much less significant but measurable amounts of MACB and ACB, respectively. It was estimated that the pooled blood contained approxi-

mately 0.19 mcg./ml. of diazepam and 0.13 mcg./ml. of Ro 5-2180, whereas Ro 5-5345 (0.008 mcg./ml.) and Ro 5-6789 (0.004 mcg./ml.) were present and/or extractable in trace amounts as the free hydroxy compounds only after incubation with Glusulase. Of these four compounds only diazepam and Ro 5-2180 were directly extracted and quantized by the GLC procedure, the other two being present as nonextractable conjugated glucuronides in the blood, which were only released as the free hydroxy compounds after incubation with Glusulase enzyme.

Studies on the metabolism of diazepam in the dog (11) have indicated a metabolic pathway in this species which is similar to man. However, when rabbits were fed chronic doses of diazepam (600–800 mg./Kg.), Jommi *et al.* (12) detected the presence of two other possible metabolites in addition to those found in man and in the dog. These were identified after strong acid hydrolysis as 2 methyl amino-5-chloro-4'-hydroxybenzophenone and 2 amino-5-chloro-4'-hydroxybenzophenone, being derived from their respective 1,4-benzodiazepines. Metabolites corresponding to these two compounds have not yet been reported in man or dog.

The findings of Schwartz *et al.* (4), on the metabolism of diazepam in man using tritiated diazepam labeled in the C₃ phenyl ring, were verified in man using TLC and GLC as analytical techniques with diazepam in pharmaceutical formulations used as the vehicle of administration. The metabolic pathways of diazepam in man are summarized in Scheme II and the data obtained indicate that the principal pathway is through the *N*-demethylation of diazepam to Ro 5-2180, which is then hydroxylated at position C-3 to Ro 5-6789. There is also evidence for a hydroxylation at C-3 of diazepam to yield Ro 5-5345 which could then be *N*-demethylated to give Ro 5-6789. The 3-hydroxy compounds are excreted as conjugated glucuronides, whereas the *N*-demethylated

metabolite Ro 5-2180 is excreted as the intact compound. No measurable amounts of intact diazepam have yet been found to be excreted in human urine, and the 3-hydroxy compound Ro 5-6789 (oxazepam) (13, 14) is a major urinary metabolite of diazepam in man.

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Polarography of Various *N*-Alkyl-*N*-nitrosoureas

By EDWARD R. GARRETT and ANTHONY G. CUSIMANO

The irreversible polarographic reductions of various *N*-alkyl-*N*-nitrosoureas were studied from a pH of 1.1–6.7. Below pH 3.2, the limiting current, i_{lim} , of *N*-methyl-*N*-nitrosourea (NNMU) is controlled by diffusion. Above this pH, adsorption or chemical reaction is the electrode-controlling process and buffer effects are observed. Similar results were obtained for *N*-ethyl-*N*-nitrosourea (NNEU) and 1,3-dimethyl-1-nitrosourea (SRI-1384), except that the buffer effects were greatly diminished for the latter compound. The acetate buffer effects on i_{lim} have been quantified and have been attributed either to adsorption of the nitrosourea (NNMU) and the buffer components onto the microelectrode, or to a reaction between these species. The polarographic method offers a sensitive (1×10^{-6} M) and reliable (0.91 per cent standard error among days) assay procedure for the nitrosoureas. All of the compounds studied in this series were more easily reduced than the parent methyl compound. A correlation between the change in the half-wave potentials and the Taft substituent constants (σ^*) was noted for simple alkyl groups such as methyl, ethyl, butyl, etc. A correlation between the half-wave potentials and the apparent first-order rate constants (k , sec.⁻¹) for hydroxyl ion catalyzed solvolysis was noted for several of the compounds studied.

THE ANTILEUKEMIC and antiviral activities of various *N*-nitrosoureas have been reported (1–4). They have a high degree of lipid solubility, are essentially nonionized, and are not bound to any extent by plasma proteins.

Degradation studies (5) of the broad spectrum antibiotic streptozotocin indicated that it is a substituted *N*-methyl-*N*-nitrosourea, and the maintenance of the biological activity could be related to the stability of the *N*-nitroso group.

A physicochemical parameter which could be correlated with biological activity, chemical structure, or reactivity would be useful in molecular modification to obtain a better drug. Al-

though the mechanism of action of the *N*-alkyl-*N*-nitrosoureas is not known for certain, it is plausible that they may act as alkylating agents since a diazoalkane may be a solvolytic intermediate (6) or that they may act *via* a redox mechanism. The polarographic half-wave potential ($E^{1/2}$) is a good electrochemical measure of the ease of reduction of such compounds.

Polarographic studies of *N*-nitrosoureas have been limited (5, 7) and have primarily been used to study the degradation of streptozotocin (5) and *N*-methyl-*N*-nitrosourea (7). This is in contrast to the detailed work on the polarographic reductions of various *N*-nitrosamines which have been studied by many investigators (8–14).

The purposes of this investigation were to characterize polarographically the reduction of a series of *N*-alkyl-*N*-nitrosoureas, to develop analytical methods, and to test for correlations of half-wave potentials with solvolytic rate constants and with substituent constants.

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