Determination of Bromazepam in Blood by Electron-Capture GLC and of Its Major Urinary Metabolites by Differential Pulse Polarography

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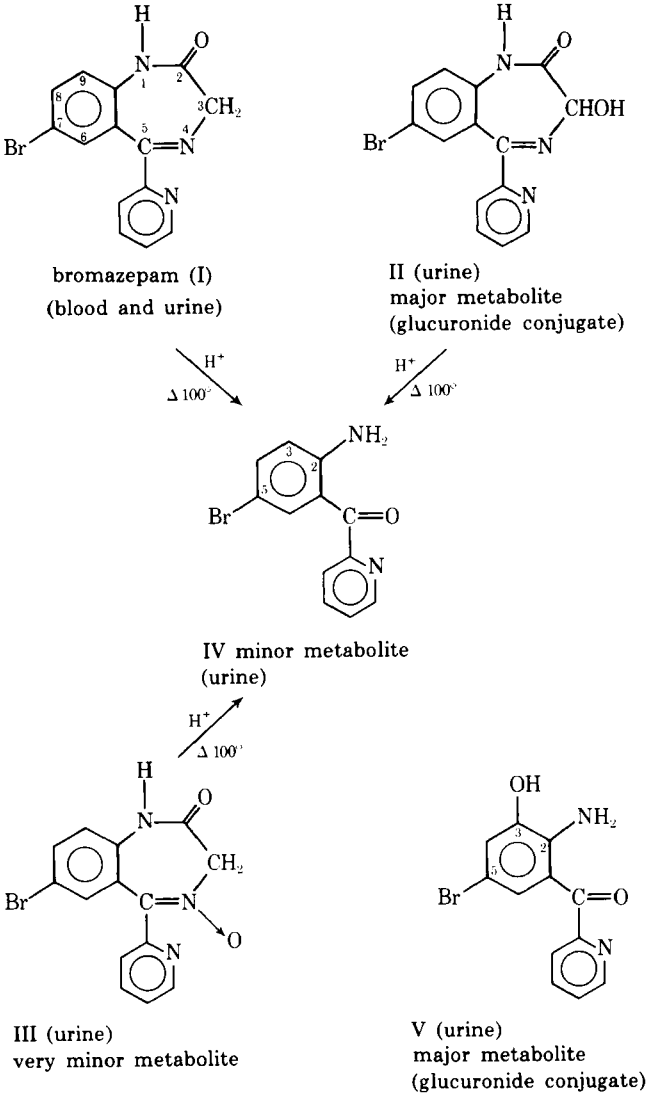
Abstract □ A sensitive and specific electron-capture GLC assay was developed for the determination of bromazepam (I), 7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one, in blood. The overall recovery of I from blood is 85 ± 5.0%, and the sensitivity limit of detection is 5–10 ng I/ml blood. In the determination of the urinary excretion, the major urinary metabolites, 3-hydroxy-bromazepam (II) and 2-amino-3-hydroxy-5-bromobenzoylpyridine (V), are selectively extracted into ether from urine buffered to pH 9.0 after incubation with glucuronidase–sulfatase, whereas I and 2-amino-5-bromobenzoylpyridine (IV) are directly extractable from urine into ether at pH 9.0. The residues of the respective ether extracts are dissolved in 1.0 *M* phosphate buffer (pH 5.5) and analyzed by differential pulse polarography, which yields two distinct peaks for the benzodiazepin-2-one and the benzoylpyridine component in each fraction. The overall recovery of I and IV is 80 ± 5.0% with sensitivity limits of 100 and 50 ng/5 ml urine, respectively; the recovery of II and V is about 45 ± 5.0% with sensitivity limits of 100 ng each/5 ml urine analyzed. Blood levels of bromazepam and the urinary excretion of the unchanged drug and its major metabolites were determined in humans following the administration of single 12-mg oral doses.

Keyphrases □ Bromazepam—electron-capture GLC analysis in blood, differential pulse polarographic analysis of major urinary metabolites □ GLC, electron capture—analysis, bromazepam in blood □ Differential pulse polarography—analysis, bromazepam urinary metabolites

Bromazepam, 7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one (I), is a member of the 1,4-benzodiazepine class of compounds and was synthesized by Fryer *et al.* (1). The pharmacology of the compound has been extensively described (2–4), and it is of clinical interest as an antianxiety agent (5–8).

The properties of I and the major known metabolites are given in Table I, and their chemical structures and reactions are given in Scheme I. Studies on the biotransformation of I in dogs, mice, and humans (9) and in dogs, rats, and rabbits (10, 11) showed that the compound was mainly metabolized by hydroxylation and hydrolysis, producing significant amounts of 3-hydroxy-bromazepam (II) and 2-amino-3-hydroxy-5-bromobenzoylpyridine (V). The parent drug (I) and 2-amino-5-bromobenzoylpyridine (IV) were excreted in lesser amounts in the urine, whereas the 4-*N*-oxide (III) was isolated as a urinary metabolite only in the dog (9). A sensitive electron-capture GLC assay for the determination of bromazepam in blood, employing acid hydrolysis to the benzoylpyridine derivative (IV) (12), was not specific for bromazepam since metabolites II and III, if present in blood, would also yield the same hydrolysis product (Scheme I) and would interfere with the specificity of the assay. An improved assay was developed for the simultaneous determination of bromazepam and its 3-hydroxy metabolite (II) as the intact 1,4-benzodiazepin-2-ones by a modification of published procedures (13, 14). When using this modified procedure, it was shown that II was not measurable in the blood of subjects chronically treated with bromazepam. Although this procedure was specific for I, it was time consuming; therefore, a simpler assay was developed which measures only bromazepam and excludes metabolites II, III, IV, and V. The method involves ether extraction of I at pH 9.0 from whole blood after protein precipitation. The metabolites are coprecipitated with the proteins and are not recovered. The



Scheme I—Chemical reactions of bromazepam and its known metabolites

Table I—Chemical Names and Physical Properties of the Compounds Referred to in Scheme I and in the Text

Compound	Chemical Name	Molecular Weight	Melting Point
I	7-Bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one (bromazepam)	316.16	237–238.5° dec.
II	7-Bromo-1,3-dihydro-3-hydroxy-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one	332.2	198–200°
III	7-Bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one 4-oxide	332.2	263° dec.
IV	2-Amino-5-bromobenzoylpyridine	277.12	97.5–99°
V	2-Amino-3-hydroxy-5-bromobenzoylpyridine	293.12	190–196°
VI	7-Bromo-1,3-dihydro-1-methyl-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one (methylbromazepam, reference standard for electron-capture GLC analysis)	330.19	135.5–137°

electron-capture GLC assay employs OV-17 as the liquid phase, which can resolve the intact drug (I) from its major metabolites (II, IV, and V), even if they are recovered in trace amounts. The high sensitivity of the ^{63}Ni-electron-capture detector is used in the pulsed dc mode for the determination of I with nanogram sensitivity. Methyl-bromazepam (VI) (Table I) is used as the reference standard in the assay.

The electron-capture GLC assay was used in the determination of blood levels of bromazepam in humans following the administration of a single oral 12-mg dose. The urinary excretion of I and its major metabolites was determined by differential pulse polarography.

EXPERIMENTAL

Electron-Capture GLC Analysis of Bromazepam in Blood

Conditions for GLC Analysis—*Column*—The column packing was a pretested preparation containing 3% OV-17 on 60–80-mesh Gas Chrom Q[1] packed in a U-shaped 1.2-m (4-ft), 4-mm i.d. borosilicate glass column. The column was conditioned at 325° for 4 hr with "no flow" of carrier gas, followed by 12 hr at 275° with carrier flowing at 40 ml/min. The useful lifespan of such a column was about 4–5 months of continuous use.

Instrumental Conditions—A gas chromatograph[2], equipped with a ^{63}Ni-electron-capture detector containing a 15.0-mCi ^{63}Ni β-ionization source was used. Argon–methane[3] (90:10), oil pumped and dry, was used as the carrier gas, with the column head pressure adjusted to 40 psig, the flow rate adjusted to 110 ml/min, and the detector purge gas adjusted to 30 ml/min. The temperature settings were as follows: oven, 245°; injection port, 270°; and detector, 325°. The conditions of column head pressure, flow rate, and oven temperature were adjusted so as to obtain retention times of 6.0 and 4.0 min for I and the reference standard (VI), respectively. A typical chromatogram is shown in Fig. 1. The solid-state electrometer[4] input was set at 10^2 and the output attenuation was 32, giving a response of 3.2×10^{-9} amp for full-scale deflection (fsd); the chart speed was 76.2 cm (30 in.)/hr, and the time constant on the 1.0-mv recorder[5] was 1 sec (fsd). The response of the ^{63}Ni-electron-capture detector (operated in the pulsed dc mode) to I showed maximum sensitivity at 60 v dc with a 150-μsec pulse rate and a 10-μsec pulse width. Under these conditions, 15 ng of I gives nearly full-scale deflection on the 1.0-mv recorder. The minimum detectable amount of I is 5.0–10.0 ng/ml of blood.

Preparation of Standard Solutions—The respective benzodiazepin-2-ones that are required as analytical standards are listed in Table I. Weigh out 10.0 mg of I and the reference standard (VI) into separate 10-ml volumetric flasks, dissolve, and dilute to volume with absolute ethanol to give stock solutions (A and A′) containing 1 mg/ml. Make suitable dilutions of A and A′ in benzene–ethanol–acetone (80:10:10) to prepare working standard solutions (B_1–B_4) in separate 10-ml volumetric flasks containing 25, 50, 75, or 100 ng of I and 50 ng of VI (added as the reference standard for electron-capture GLC analysis) per 0.1 ml of solution. Add 1 g of anhydrous sodium sulfate to these solutions to maintain their anhydrous state.

Aliquots (10 μl) of solutions B_1–B_4 are injected into the chromatograph to optimize the electron-capture detector response to I and VI and to establish the conditions for GLC analysis; 100-μl aliquots of these standard solutions are also added to control blood as the internal standards, which are used to determine the overall recovery of I and as the calibration curve for the quantitation of unknowns (Fig. 2).

After the electron-capture GLC conditions have been optimized

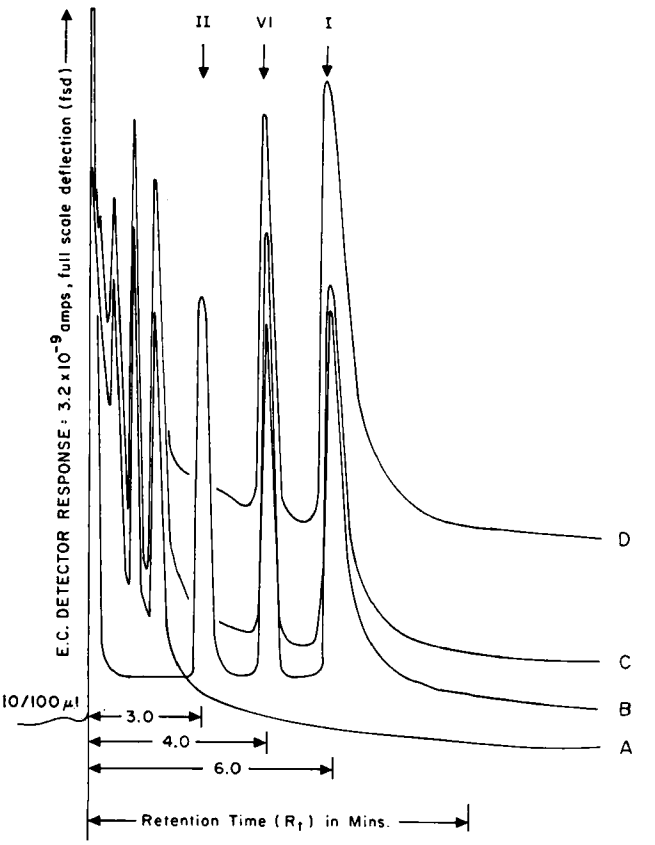


Figure 1—*Chromatograms of: (A) control blood extract; (B) authentic standards of I, II, and VI; (C) internal standard of 10 ng of I and 5 ng of methyl-bromazepam (reference standard) recovered from control blood; and (D) patient blood extract after 12-mg oral dose of I.*

[1] Applied Science Labs., Inc., State College, Pa.
[2] Micro-Tek model MT-220, Tracor Instruments, Austin, Tex.
[3] Matheson.
[4] Model 8169, Tracor Instruments, Austin, Tex.
[5] Model 194, Honeywell Instruments, Fort Washington, Pa.

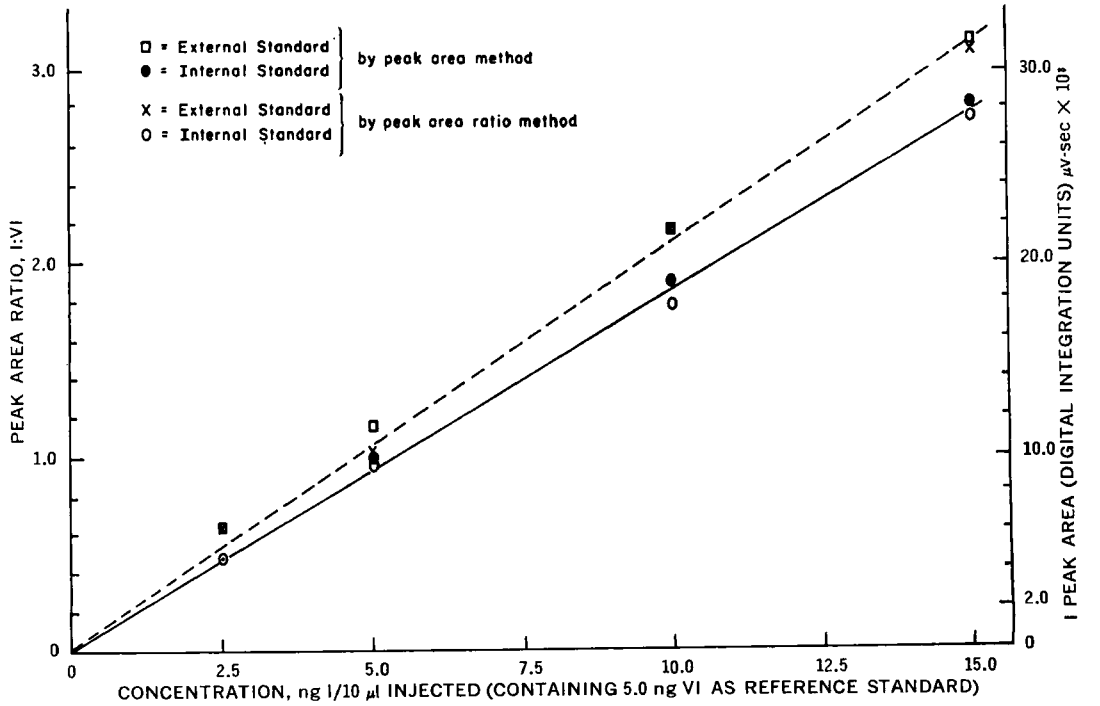


Figure 2—*Electron-capture detector calibration curves for I using either the direct calibration method [peak area (square centimeters)* versus *concentration (nanograms)] or the relative calibration method [peak area ratio of bromazepam–methyl-bromazepam (reference standard)* versus *concentration (nanograms)].*

using the pure authentic compound, the external calibration curve is prepared *after* the injection of a 2 × 10-μl aliquot of the control blood extract. *In practice,* 10-μl injections of the external standards are made *between every two or three consecutive biological samples.* The external standard curve is required only for the determination of percent recovery.

Reagents—All reagents were of analytical (ACS certified) reagent grade purity (>99%) and were used without further purification. All inorganic reagents were made up in double-distilled water. These included 1 *M* H_3BO_3–Na_2CO_3–KCl buffer (pH 9.0) prepared as previously described (12), 2 *N* H_2SO_4, and 2 *N* NaOH. Ether[6], benzene[6], acetone[7], methanol[7], ethanol[8], and a mixture of benzene–ethanol–acetone (80:10:10) (stored over anhydrous sodium sulfate) were the other reagents used.

Extraction of Blood for Determination of Bromazepam—Into a 50-ml centrifuge tube, add 2 ml of oxalated whole blood and 2 ml of distilled water, and mix well by inversion. Add 5 ml of 2 *N* H_2SO_4 dropwise and slurry vigorously on a vortex supermixer to precipitate the protein. Along with the samples, process four 2-ml specimens of control blood to which 25, 50, 75, or 100 ng of I (100 μl of solutions B_1–B_4 evaporated to dryness under nitrogen) has been added as the internal standard. The acidified specimen (including the precipitate) is washed with 2 × 15-ml portions of ether[9] by shaking for 5 min on a reciprocating shaker and centrifuged, and the supernatant ether layer is aspirated off and discarded. The ether-washed sample is then neutralized with 5.0 ml of 2 *N* NaOH, buffered with 2 ml of pH 9.0 borate buffer, mixed well on a supermixer, and adjusted to pH 9.0 with dilute alkali. The samples are then extracted with 2 × 10 ml of ether, centrifuged after each extraction at 2000 rpm (preferably at 0–4° in a refrigerated centrifuge), and the ether extracts are combined successively in a 15-ml conical centrifuge tube by evaporating them to dryness in the water bath of a rotary evaporator[10] at 35–40°. The residues are vacuum dried (over sodium hydroxide pellets) for 15 min and then dissolved in 100 μl of the benzene–ethanol–acetone (80:10:10) solvent mixture. A 10-μl aliquot of this solution is injected for electron-capture GLC analysis. The peaks due to bromazepam and the reference standard are identified by their respective retention times (Fig. 1), and their respective peak areas are determined either by measuring peak height (centimeters) × width at half-height (centimeters) using the slope baseline technique or by electronic digital integration.

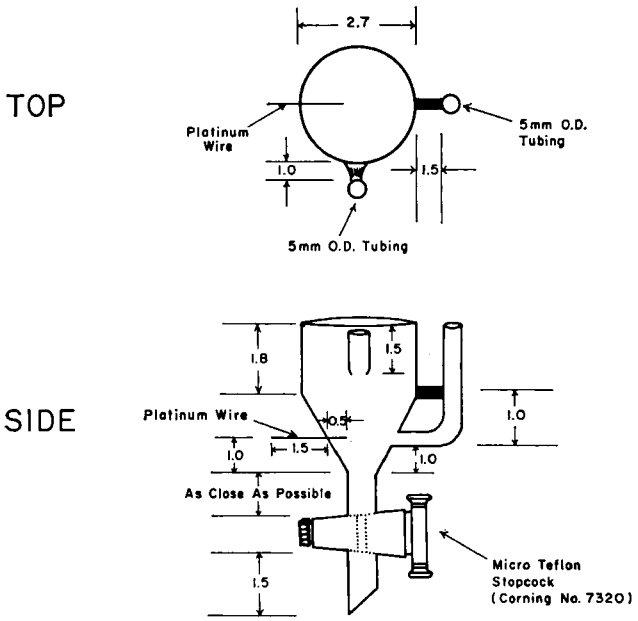


Figure 3—*Schematic diagram of the semimicropolarographic cell. (All dimensions are in centimeters except where noted, and the platinum wire used was B & S No. 22 gauge.)*

[6] Catalog No. 0848 and No. 1043, respectively; Mallinckrodt Chemical Works, St. Louis, Mo.

[7] Catalog No. A-18 and No. A-412, respectively; Fisher Scientific Co., Fairlawn, N.J.

[8] USP, 200 proof, Publicker Industries Inc., Philadelphia, Pa.

[9] The purity of ether for electron-capture GLC analysis is determined by evaporating 20 ml to dryness, dissolving the residue in 100 μl of benzene–ethanol–acetone (80:10:10), and injecting a 10-μl aliquot. The chromatogram must be free of interfering peaks in the retention area following 2 min after injection.

[10] Buchler Evapomix, Buchler Instruments, Fort Lee, N.J.

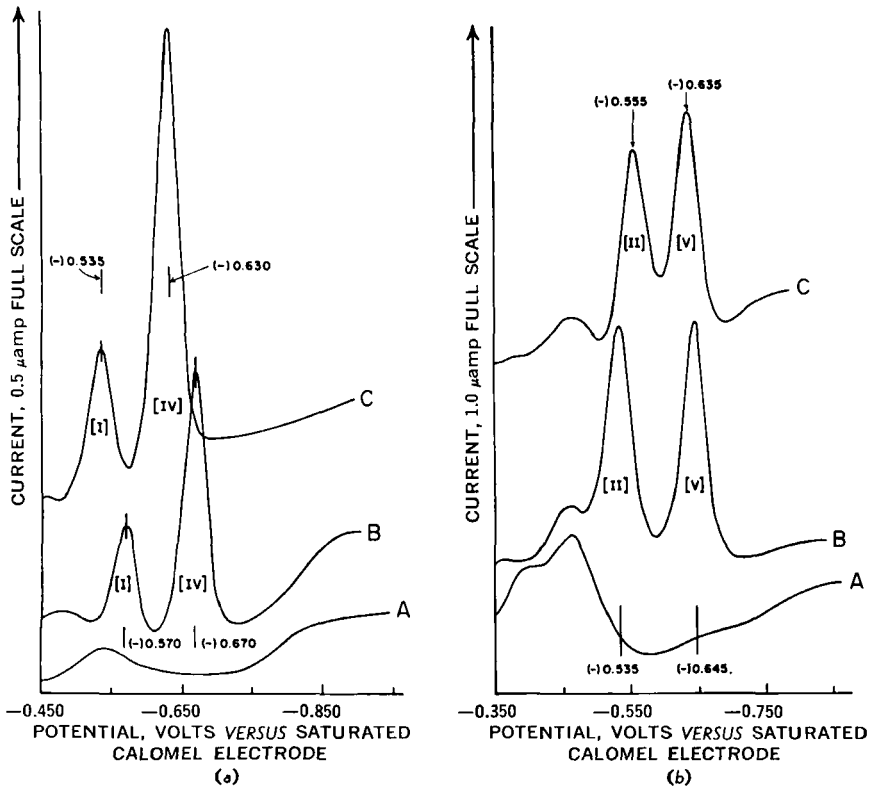


Figure 4—*Differential pulse polarograms of:* (a) *I and IV and* (b) *II and V obtained in 1.0* M *pH 5.5 phosphate buffer as the supporting electrolyte. Key: A, control urine blank; B, authentic standard mixture; and C, authentic compounds recovered from urine.*

Calculations—The concentration of bromazepam in the aliquots of the unknowns injected is calculated by interpolation from the internal standard curve (Fig. 2) by using either the direct calibration (peak area *versus* concentration) or the relative calibration (peak area ratio) techniques. The sensitivity of the electron-capture GLC assay is about 5–10 ng bromazepam/ml blood assayed.

Differential Pulse Polarographic Analysis of Major Urinary Metabolites of Bromazepam

The major urinary metabolites of I in humans are the 3-hydroxy metabolite (II) and the 2-amino-3-hydroxy-5-bromobenzoylpyridine metabolite (V), which are present mainly as glucuronide–sulfate conjugates (9). Small amounts of the intact drug (I) and metabolite IV are also excreted. The urine specimen is first extracted at pH 9.0 with ether, which quantitatively removes I and IV. The specimen is then titrated to pH 5.5 and incubated with glusulase enzyme to deconjugate metabolites II and V, which are then extracted into ether after adjusting the sample to exactly pH 7.75.

Reagents—*1.0* M *Phosphate Buffer (pH 5.5)*—Dissolve 138.0 g of $NaH_2PO_4 \cdot H_2O$ in distilled water and dilute to 1 liter. Titrate carefully with 1.0 *N* NaOH to pH 5.5 and shake well by inversion to effect equilibration.

Glusulase Enzyme[11]—Use the diagnostic reagent (100,000 units glucuronidase and 50,000 units sulfatase/ml).

Procedure—Into a 50-ml centrifuge tube, add 5 ml of urine, 5 ml of 1 *M* pH 9.0 borate buffer, and 10 ml of ether for the first extraction. Along with the unknowns, process a 5-ml specimen of control urine (taken preferably from the same subject prior to medication) and three 5-ml specimens of control urine to which 200, 400, or 600 ng each of I and IV is added as the internal standard. Extract all samples by shaking for 10 min on a reciprocating shaker, centrifuge at 2000 rpm, and transfer the supernate into a 15-ml conical centrifuge tube. Reextract the sample with another 10 ml of ether and combine the extracts by serial evaporation at 35–40° in the water bath of the rotary evaporator. This series of extracts (A) contain I and IV. The urine sample is then titrated potentiometrically to pH 5 by the dropwise addition of 6 *N* HCl and is buffered to pH 5.5 with 5 ml of pH 5.5 phosphate buffer after adding 0.2 ml of glusulase (2% of total volume). Then the entire contents are transferred into a 50-ml erlenmeyer flask.

The three extracted control urine specimens are treated in the same manner and are now spiked with 1, 2, or 3 μg each of II and V, the major conjugated metabolites. Stopper all flasks loosely with cotton and place in an incubation shaker[12] and incubate at 37° for 2.5 hr with mild shaking. After incubation, cool the samples to room temperature and adjust the solution to pH 7.75 (using a pH meter) by titrating the sample dropwise with 6 *N* NaOH. Transfer this solution quantitatively into a 50-ml stoppered centrifuge tube and extract twice with 15 ml of ether by shaking for 10 min and then centrifuging for 5 min. Combine the ether extracts in a 15-ml conical centrifuge tube by successive transfer and evaporation to dryness as before. This series (B) contains the conjugated metabolites II and V. The residues of both series A and B are solubilized with 100 μl of methanol, dissolved in 2 and 4 ml, respectively, of 1 *M* phosphate buffer (pH 5.5), and deaerated for 5 min with nitrogen bubbled through the sample with a sintered-glass (fritted) tube. Two milliliters of each solution is transferred into the semimicropolarographic cell[13] (Fig. 3), which contains the three operational electrodes, and analyzed by differential pulse polarography.

Conditions for Polarographic Analysis—A polarographic analyzer[14] equipped with a drop timer[15] was used in conjunction with a three-electrode polarographic cell comprised of a dropping mercury electrode, a saturated calomel electrode, and a platinum wire as the auxiliary electrode, as previously described (15). The drop time was 1.0 sec, and the drop rate was 2.74 mg/sec, where $(m^{2/3})(t^{1/6}) = 1.958$. The current range was set at 0.5 or 1 μamp for a peak response of full-scale deflection, the scan range was 1.5 v, and the scan rate was 2 mv/sec. The samples were scanned between −0.350 and −0.700 v *versus* the saturated calomel electrode, and the polarograms were obtained on an *X–Y* recorder[16]. The peak potential, E_p, due to the reduction of the azomethine ($>C_5{=}N_4{-}$) group of I and its 3-hydroxy metabolite (II) occurs at −0.535 and −0.555 v *versus* the saturated calomel electrode, respectively, whereas the peak due to the reduction of the carbonyl

[11] Endo Laboratories, Inc., Garden City, N.Y.

[12] Dubnoff, Precision Scientific, Inc., Chicago, Ill.

[13] Designed by M. R. Hackman and M. A. Brooks, Hoffmann-La Roche Inc., Nutley, NJ 07110 (1972).

[14] PAR model 174, Princeton Applied Research Corp., Princeton, N.J.

[15] PAR model 172A, Princeton Applied Research Corp., Princeton, N.J.

[16] Houston Omnigraph model 2200-3-3, Houston Instruments, a division of Bausch & Lomb, Inc., Bellair, Tex.

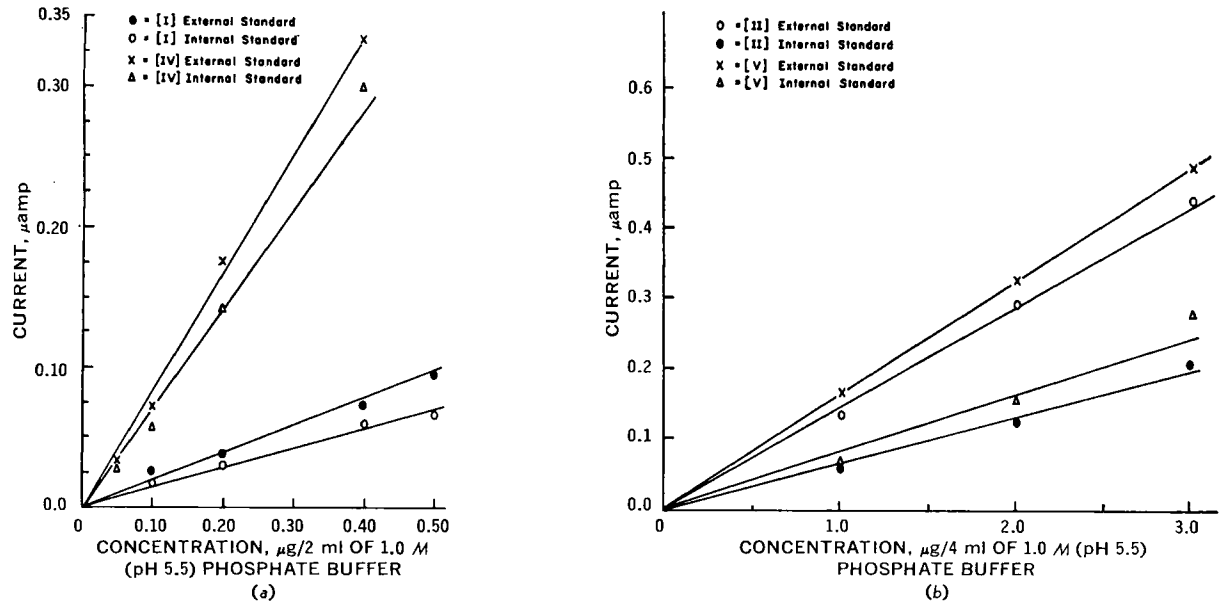


Figure 5—*Calibration curves for the differential pulse polarographic analysis of:* (a) *I and IV, and* (b) *II and V.*

(>C=O) group of metabolites, IV and V, occurs at −0.630 and −0.635 v *versus* the saturated calomel electrode, respectively (Fig. 4). The two analytical peaks are well resolved, with a peak-to-peak separation of nearly 100 mv. Therefore, series A, containing a mixture of I and IV, and series B, containing a mixture of II and V, can be analyzed and the two peaks readily distinguished.

Calculations—The current (microamperes) resulting from each peak and the overall recovery of each compound are determined as described previously (15, 16), while the concentration of each component in the unknowns is determined by interpolation from the respective internal standard curves (Fig. 5).

RESULTS AND DISCUSSION

The chromatographic analysis of bromazepam (I) and metabolite II manifested certain problems. Authentic standards of I and methyl-bromazepam (reference standard, VI), dissolved in benzene–ethanol–acetone (80:10:10) and analyzed by electron-capture GLC, indicated that although the two compounds were resolved, the peak of I showed excessive tailing. The chromatograms obtained from blood extracts containing both I and VI showed significantly improved, well-resolved Gaussian-shaped peaks for both compounds at the same retention times as before (Fig. 1), but with enhanced sensitivity to the electron-capture detector.

Table II—Blood Levels of Bromazepam in Three Human Subjects following Administration of a Single 12-mg Oral Dose

Subject	D.L.P. (m)	G.A.V. (m)	R.R.E. (m)
Weight, kg	68.10	104.42	87.62
Dose, mg/kg	0.176	0.115	0.137
Hours Postdosing	Blood Concentration, μg Bromazepam/ml		
0	—	—	—
0.5	0.12	0.05	0.13
1	0.12	0.08	0.13
2	0.12	0.11	0.13
4	0.10	0.10	0.13
6	0.10	0.10	0.12
8	0.10	0.10	0.13
12	0.09	0.07	0.13
24	0.05	0.05	0.08
30	0.03	0.03	0.06
36	0.02	0.02	0.05
48	0.01	0.01	0.02
72	N.M.[a]	N.M.	0.01

[a] N.M. = not measurable, <0.01 μg/ml.

These phenomena suggested that components in the blood extract probably form an adsorption complex with exposed active sites on the column, thereby reducing adsorption losses of I and VI and resulting in more symmetrical peaks with enhanced detector response. Consequently, pretreatment of the column by injecting control blood extracts is essential for obtaining reproducible analysis of I.

The electron-capture GLC behavior of metabolite II was not reproducible when injected as the pure solution or from a blood extract. Although authentic II was well resolved from I with a separation of about 3 min and was quite sensitive to the electron-capture detector, its overall recovery from blood (40–50%) and reproducibility (±10–15%) were unsatisfactory. Attempts at preparing the trimethylsilyl derivative of II, using mixtures of either hexamethyldisilazane and trimethylchlorosilane or bis(trimethylsilyl)acetamide in pyridine, did not yield reproducible derivatization and were not pursued further.

It has been reported that 3-hydroxy-*N*-desalkyl-1,4-benzodiazepin-2-ones such as oxazepam and lorazepam (17, 18) undergo thermolytic rearrangement during GC–mass spectrometric analysis, resulting in the formation of their respective quinazoline-carboxaldehydes. A similar thermolytic rearrangement may be responsible for the poor overall recovery and reproducibility of II during electron-capture GLC analysis. Although II is a major urinary metabolite, it is not present in blood (in the free or directly extractable form) even on chronic administration of I in any significant amount, so its quantitation can be omitted.

The N_4-oxide metabolite (III) was not isolated as a metabolite in humans or in mice, although it was excreted as a minor metabolite by the dog (9). Authentic III, when analyzed by electron-capture GLC, gave a peak with the same retention time as I. It is possible that it can undergo either a loss of oxygen to form I or a thermolytic rearrangement to give an epoxide (17) with the same retention time as I. Since this compound is not recovered in the final sample extract, it does not interfere with the determination of I. The benzoylpyridines IV and V, even if recovered in trace amounts, are eluted with the interfering peaks of the solvent front and cannot be quantitated under these conditions. The electron-capture GLC assay is, therefore, specific for the intact drug.

Differential pulse polarography has been successfully applied to the determination of several types of drugs in their intact form such as benzodiazepines (19, 20), the nitroimidazoles (21), and pyrimidine-containing compounds (16). Others such as glibornuride (a tolylsulfonylurea) (15), phenobarbital, and diphenylhydantoin (22) were determined as their nitro derivatives. The 1,4-benzodiazepin-2-ones and their benzophenones can be quantitated in the submicrogram range due to the ease of reduction of the (>C_5=N_4—) azomethine group of the former and the (>C=O) carbonyl group of the latter in dilute acids and to the relatively high sensi-

Table III—Excretion (0–72 hr) of Bromazepam and Its Major Urinary Metabolites in Humans Determined by Differential Pulse Polarography

Subject	Percent of Dose Excreted as				Dose Administered = 12 mg	
	I	II	IV	V	mg Recovered	% of Dose
D.L.P.	2.5	22.6	0.35	46.6	8.65	72.1
G.A.V.	1.9	27.8	0.75	29.9	6.91	57.6
R.R.E.	1.8	34.8	0.21	39.5	9.19	76.6

tivity (microamperes per microgram) achieved. Consequently, I, II, IV, and V can be selectively extracted as mixtures of I and IV and of II and V, successively, and quantitated with a minimum of cleanup because the coextracted impurities do not interfere in the potential region of interest. The use of the semimicropolarographic cell (20) (Fig. 3) with a minimum sample volume of 2 ml, a longer drop time of 1 sec, and a slower scan speed of 2 mv/sec contributed to increasing the reduction current generated and, consequently, the overall sensitivity of the assay.

The polarographic analysis of a mixture of authentic standards of I and IV (Fig. 4*a*) and of II and V (Fig. 4*b*) in 1.0 *M* pH 5.5 phosphate buffer shows two well-resolved analytical peaks for I at −0.570 v and IV at −0.670 v *versus* the saturated calomel electrode and for II at −0.535 v and V at −0.645 v *versus* the saturated calomel electrode (polarogram B). The same compounds recovered from urine again showed well-resolved peaks but with different E_p potentials. The peak potential E_p for I, IV, and V showed a shift toward a more positive potential, with the peak for I now at −0.535 v, for IV at −0.630 v, and for V at −0.635 v *versus* the saturated calomel electrode, whereas the peak for II shifted to a more negative potential at −0.555 v *versus* the saturated calomel electrode (polarogram C). These peak shifts are probably due to physicochemical interactions with impurities extracted from control urine (polarogram A). The calibration curves of authentic (external standards) of I, II, IV, and V and of the same compounds as internal standards recovered from urine (Fig. 5) show the linearity of the assay and its higher sensitivity for the benzoylpyridine metabolites IV and V, respectively.

The overall recovery of I and IV, which are extracted directly into ether from urine buffered to pH 9.0, was 80 ± 5.0%. No measurable amounts of II and V were present in this extract, as determined by electron-capture GLC analysis. Therefore, any interference is minimal and can be neglected.

Metabolites II and V are extracted after enzymatic deconjugation and analyzed as a mixture (Fig. 4*b*). Although the recovery of II and V into ether from urine buffered to pH 7.75 was only 45 ± 5.0%, the concentration of each present in the urine of treated subjects was sufficiently high for accurate quantitation. The overall recovery can be increased significantly (>75%) by using either ethyl acetate or methylene chloride (dichloromethane). It is achieved, however, at the expense of greatly increased impurities coextracted with the compounds of interest and precludes polarographic analysis without extensive cleanup of the sample.

Blood Levels of Bromazepam in Humans Determined by Electron-Capture GLC Analysis—Blood levels were determined in three subjects (in a pilot study) following the oral administration of a single 12-mg dose of the clinically used tablet formulation. The blood data (Table II) indicate the presence of measurable levels from 30 min to 72 hr postdosing, thereby demonstrating the clinical utility of the method. The assay was used to determine the blood levels in humans following single 12-mg oral doses and chronic administration of oral doses ranging from 3 to 9 mg/day over a 30-day dosing period in the evaluation of the pharmacokinetic profile of the drug in humans[17]. Blood specimens analyzed for II indicated that it was not present in any significant amounts following both single and chronic oral dosing.

Urinary Excretion of Bromazepam and Its Major Metabolites—The urinary excretion of bromazepam and its major metabolites following single 12-mg oral doses was determined by differential pulse polarography (Table III). The data indicate that II and V were the predominant metabolites excreted, accounting for a total of about 58–77% of the administered dose, along with lesser amounts of the intact drug (I) and its benzoylpyridine derivative (IV) over the 72-hr excretion period.

[17] S. A. Kaplan *et al.*, Hoffmann-La Roche Inc., Nutley, NJ 07110, unpublished data on file.

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