the presence of a highly structured, nonpolar system, similar to surfactant micelles in a polar medium.

Further ramifications are currently under study in these laboratories.

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ˣ To whom inquiries should be directed.

Electron-Capture GLC Determination of Blood Levels of 7-Chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one in Humans and Its Urinary Excretion as Lorazepam Determined by Differential Pulse Polarography

J. ARTHUR F. de SILVA ˣ, IHOR BEKERSKY, and MARVIN A. BROOKS

Abstract □ This report presents data to demonstrate the clinical utility of a previously published electron-capture GLC assay in the measurement of blood levels of 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one in humans following a single oral 4-mg dose. It also describes a differential pulse polarographic assay for the quantitation of its major urinary metabolite, lorazepam, in humans. This assay has greater sensitivity and specificity than the previously published Bratton–Marshall spectrophotometric assay.

Keyphrases □ 7-Chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one—electron-capture GLC determination of human blood levels □ Lorazepam—urinary metabolite of 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one, determined by differential pulse polarography □ GLC, electron capture—determination, 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one in human blood samples □ Differential pulse polarography—determination, lorazepam as urinary metabolite of 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one in humans

The 1,4-benzodiazepine class of compounds are clinically important as tranquilizers, hypnotics, and muscle relaxants (1). 7-Chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one (I) was synthesized by Sternbach *et al.* (2) and is currently undergoing clinical evaluation as an antianxiety agent. A sensitive and specific electron-capture GLC assay for the determination of I and its major metabolite, lorazepam (II), was published previously (3)[1].

This report presents data to demonstrate the clinical utility of the electron-capture GLC assay in the measurement of blood levels of I in humans following a single oral 4-mg dose. It also describes a differential pulse polarographic assay for the quantitation of lorazepam. The polarographic analysis of oxazepam and lorazepam in pharmaceutical formulations was described previously (4, 5).

EXPERIMENTAL

Clinical Protocol—A pilot blood level study was conducted in three subjects[2] following the administration of a single 4-mg oral dose (as 2 × 2-mg tablets), and whole blood specimens were collected as indicated in Table I. Urine specimens were collected as indicated in Table II. The oxalated blood specimens and urine specimens were stored frozen until analyzed.

Analysis of Specimens—Blood and urine levels of I were determined by the published electron-capture GLC assay (3), which has sensitivity limits of 0.5–1.0 ng/ml for I and of 4–5 ng/ml for II in blood or urine using a 15-mCi nickel-63 electron-capture detector. The major urinary metabolite in humans (lorazepam) is present mainly as a glucuronide conjugate (6). It is extracted from urine following deconjugation with glucuronidase–sulfatase and is analyzed by differential pulse polarography (Fig. 1), with a sensitivity limit of 50 ng/ml for II in urine using a 5-ml specimen/analysis.

Differential Pulse Polarographic Analysis of II—Lorazepam (II) is present in the urine mainly as a glucuronide and/or sulfate conjugate; small amounts of I are also excreted. The urine specimen is first extracted at pH 9.0 with ether[3], which quantitatively removes I and any unconjugated lorazepam, and is processed for electron-capture GLC analysis (3). The specimen is then titrated to pH 5.3, incubated with glusulase enzyme[4] (2% by volume) to deconjugate II, and then extracted into ether after adjusting the pH to 9.0 as previously described (3).

Internal standards of 200, 400, or 1000 ng of II are added to the control urine specimens prior to incubation and are carried through the entire procedure. After incubation, the samples are cooled to room temperature and the pH is adjusted to 9.0 using a pH meter by titrating the sample dropwise with 6 *N* NaOH. This

[1] Consult Fig. 1 and Table I in this reference for the chemical structures and nomenclature of Compounds I, II, and V.

[2] Conducted under the supervision of Dr. James D. Moore at the Deer Lodge Research Unit, Deer Lodge, Mont.

[3] Diethyl, absolute, analytical reagent grade, Mallinckrodt.

[4] Endo Laboratories, Garden City, Long Island, N.Y.

Table I—Blood Levels[a] of *I* in Humans following a Single Oral Dose in a Tablet Formulation

Time	Subject 1 (68.1 kg), Dose of 0.059 mg/kg	Subject 2 (68.1 kg), Dose of 0.059 mg/kg	Subject 3 (73.6 kg), Dose of 0.054 mg/kg
	ng of I/ml of Blood		
15 min	2.0	1.8	0.7
30 min	4.0	6.0	3.4
1 hr	4.4	7.7	5.6
2 hr	4.8	15.5	5.9
4 hr	7.4	12.8	5.7

[a] Determined by electron-capture GLC analysis. Sensitivity limit = 0.5–1.0 ng of I/ml of blood.

solution is transferred quantitatively into a 50-ml centrifuge tube and extracted twice with 15 ml of ether by shaking for 10 min and then centrifuging for 5 min.

The ether extracts are combined in a 15-ml conical centrifuge tube by successive transfer and evaporation to dryness. The residues are solubilized with 100 μl of methanol, dissolved in 2 ml of 0.1 *N* HCl, and deaerated for 1 min with nitrogen bubbled through the sample with a sintered-glass (fritted) tube. Then the solution is transferred into a semimicropolarographic cell and analyzed by differential pulse polarography.

Conditions for Polarographic Analysis—A polarographic analyzer[5] equipped with a drop timer[6] was used in conjuction with a three-electrode semimicropolarographic cell comprised of a dropping mercury electrode, a saturated calomel electrode, and a platinum wire as the auxiliary electrode, as previously described (7). The drop time was 1.0 sec, and the drop rate was 2.74 mg/sec where $(m^{2/3})(t^{1/6}) = (1.958\ mg^{2/3})(sec^{-1/2})$.

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

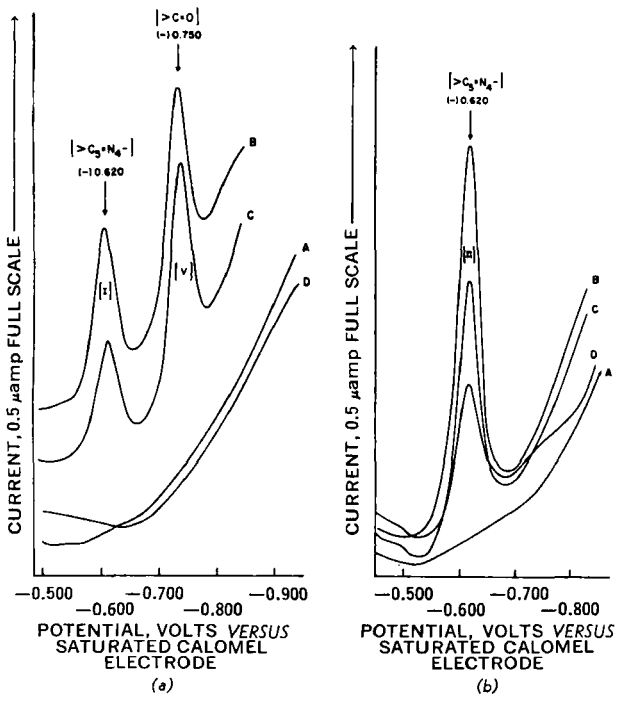
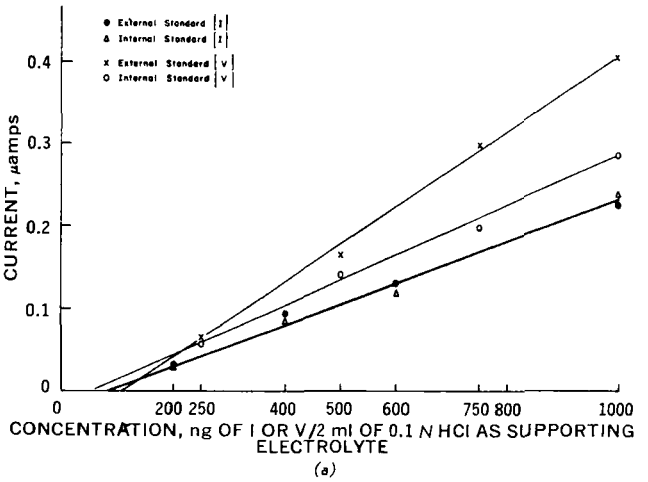


Figure 1—*Differential pulse polarograms:* (a) *Compound I and its benzophenone* (*V*), *and* (b) *II obtained in 0.1* N *HCl as the supporting electrolyte. Key: A, control urine blank; B, authentic reference standards; C, authentic standards recovered from urine; and D, patient urine, 0–6 hr postdosing fraction.*



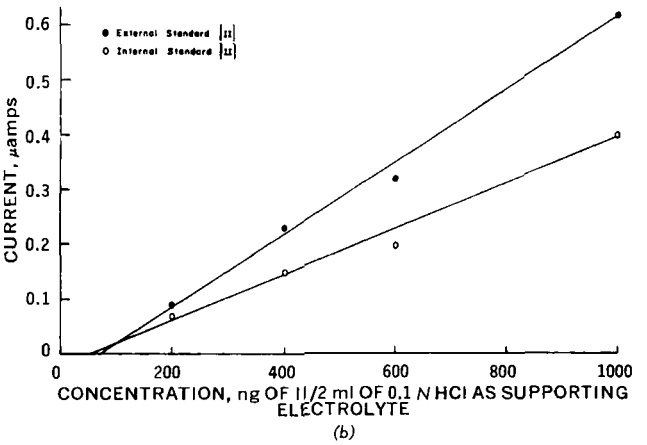


Figure 2—*Calibration curves for the differential pulse polarographic analysis:* (a) *Compound I and its benzophenone* (*V*), *and* (b) *II.*

was 2 mv/sec. The samples were scanned between −0.500 and −0.900 v *versus* the saturated calomel electrode and the polarograms were obtained on an X–Y recorder[7]. The analytical peak due to the reduction of the azomethine (>C_5=N_4—) group of II occurs at −0.620 v *versus* the saturated calomel electrode (Fig. 1*b*).

Calculations—The current (microamperes) resulting from II and the overall recovery of II are determined as described previously (8, 9). The concentration of II in the unknowns is determined by interpolation from the internal standard curve (Fig. 2*b*).

RESULTS AND DISCUSSION

Differential pulse polarography has been successfully applied to the determination of several classes of drugs in biological fluids (10) in their intact form such as benzodiazepines (7), nitroimidazoles (11), and pyrimidine-containing compounds (9); others such as glibornuride (a tolylsulfonylurea) (8), phenobarbital, and diphenylhydantoin (12) have been determined as their nitro derivatives. The 1,4-benzodiazepin-2-ones can be quantitated in the submicrogram range due to the ease of reduction of the azomethine group in dilute acids and the relatively high sensitivity (microamperes per microgram) achieved.

Consequently, I and II can be selectively extracted and quantitated with a minimum of cleanup because the coextracted impurities do not interfere in the potential region of interest. The use of a semimicropolarographic cell (7), with a minimum sample volume of 2 ml compared to that of 5 ml with the original cell (9), and the

[5] PAR model 174, Princeton Applied Research Corp., Princeton, N.J.
[6] PAR model 172A, Princeton Applied Research Corp., Princeton, N.J.
[7] Omnigraph model 2200-3-3, Houston Instruments, Bellaire, Tex.

Table II—Urinary Levels of I and II-Glucuronide in Humans

Excretion Period, hr	Subject 1		Subject 2		Subject 3	
	Total Excreted as I[a], μg	Total Excreted as II[b], μg	Total Excreted as I[a], μg	Total Excreted as II[b], μg	Total Excreted as I[a], μg	Total Excreted as II[b], μg
0–6	1.94	16.5	1.01	14.8	1.49	30.8
6–12	1.86	27.2	2.46	35.0	0.79	44.1
12–24	2.95	75.5	3.54	81.6	0.99	41.8

[a] Determined by electron-capture GLC as free or directly extractable drug. [b] Determined by differential pulse polarography after deconjugation with glucuronidase–sulfatase.

use of a longer drop time of 1 sec and a slower scan speed of 2 mv/sec have all increased the reduction current generated and, therefore, the overall sensitivity of the assay.

Both I and II can be quantitated in the 150–250-ng range by differential pulse polarography at a reduction potential (E_p) equal to −0.620 v *versus* the saturated calomel electrode, with an overall recovery of 95 ± 5.0% for I and 65 ± 5.0% for II, using ether as the extracting solvent.

The overall recovery of II can be increased significantly (>85%) by using either ethyl acetate or methylene chloride (dichloromethane); however, it is achieved at the expense of greatly increased impurities being coextracted with the compounds of interest and precludes direct polarographic analysis without extensive cleanup of the sample.

Differential pulse polarographic analysis of I and its benzophenone (V) as a mixture is possible because of the separation of the reduction peaks of the azomethine group of I at −0.620 v *versus* the saturated calomel electrode and of the carbonyl group of V at −0.750 v *versus* the saturated calomel electrode (Fig. 1*a*). They can be quantitated at concentrations greater than 150 ng of each/2 ml of 0.1 *N* HCl as the supporting electrolyte (Fig. 2*a*).

Blood specimens obtained over 4 hr following the oral administration of a single 4-mg dose of I were analyzed by electron-capture GLC (3) (Table I). The drug was measurable throughout the 15-min to 4-hr postdosing interval, demonstrating the clinical utility of the assay. No measurable level of unconjugated lorazepam (II) was seen in the blood at any time interval. 2-Amino-5,2′-dichlorobenzophenone (V) and 4-(2-chlorophenyl)-6-chloroquinazolin-2(1*H*)-one (IV) (3), which were reported to be minor metabolites of II in humans (13), were not detected by electron-capture GLC in this study.

The urinary excretion of I and II in humans was determined by electron-capture GLC and differential pulse polarographic analysis, respectively (Table II).

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[x] To whom inquiries should be directed.