Determination of Clonazepam and Flunitrazepam in Blood and Urine by Electron-Capture GLC

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Abstract □ A sensitive and specific electron-capture GLC assay was developed for the determination of clonazepam and flunitrazepam (two members of the 7-nitro-1,4-benzodiazepin-2-one class of compounds) in blood and urine. The assay for clonazepam uses flunitrazepam as a reference standard and vice versa. The assay for both compounds involves selective extraction into ether from blood or urine at pH 9.0 followed by acid hydrolysis to their respective benzophenones, which are extracted and quantitated by electron-capture GLC. Recovery from blood or urine is quantitative, and the sensitivity limits of the assay are 0.5–1.0 ng/ml of specimen analyzed. The assay was successfully applied to the disposition of clonazepam in man following single 2-mg oral doses and of flunitrazepam in the dog following intravenous and oral doses of 2 mg/kg. A differential pulse polarographic assay for the determination of the major urinary metabolites of clonazepam is also presented.

Keyphrases □ Clonazepam—electron capture GLC determination in biological specimens □ Flunitrazepam—electron-capture GLC determination in biological specimens □ Benzodiazepines, clonazepam and flunitrazepam—electron-capture GLC determination in biological specimens □ GLC, electron-capture detection—analysis, clonazepam and flunitrazepam in biological specimens

The 1,4-benzodiazepine class of compounds has shown marked anticonvulsant properties in addition to their antianxiety and muscle relaxant properties (1, 2), and these compounds are among the more widely prescribed minor tranquilizers and anticonvulsant drugs (3). The 7-nitro-1,4-benzodiazepin-2-ones (nitrazepam, clonazepam, and flunitrazepam) (4) have shown marked anticonvulsant properties in several animal species (1, 2, 5–10). Clonazepam is clinically effective in controlling minor motor seizures (petit mal) at doses of 1–2 mg/day orally (11–17), while flunitrazepam is a clinically potent hypnotic (18, 19) and anesthetic induction agent (20, 21).

Clonazepam[1] [7-nitro-5-(2-chlorophenyl)-1,3-dihydro-(2*H*)-1,4-benzodiazepin-2-(1*H*)-one, A] (Scheme I and Table I), flunitrazepam [7-nitro-5-(2-fluorophenyl)-1,3-dihydro-1-methyl-(2*H*)-1,4-benzodiazepin-2-one, I] (Scheme I and Table II), and *N*-desmethyl-flunitrazepam (Compound II in Table II) can be hydrolyzed quantitatively in acid to their benzophenones (22) (Scheme I), 2-amino-5-nitro-2′-chlorobenzophenone (Compound J in Table I), 2-methylamino-5-nitro-2′-fluorobenzophenone (Compound V in Table II), and 2-amino-5-nitro-2′-fluorobenzophenone (Compound VI in Table II), respectively. Nitrazepam[2] [7-nitro-5-phenyl-1,3-dihydro-(2*H*)-1,4-benzodiazepin-2-(1*H*)-one, G] (Scheme I and Table I) is hydrolyzed to 2-amino-5-nitrobenzophenone (Compound H in Table I and Scheme I). These benzophenones are quantitated by the highly sensitive GLC assay described.

Attempts at determining clonazepam and flunitrazepam as the intact benzodiazepin-2-ones were unsuccessful. Clonazepam gave a very broad and poorly defined peak by electron-capture GLC on the phenyl silicone phases OV-1 and OV-17, whereas flunitrazepam was eluted as a symmetrical Gaussian-shaped peak with sufficient sensitivity for quantitation in the nanogram range. However, flunitrazepam and its *N*-desmethyl metabolite (II), unlike clonazepam, are unstable in acid (Table II). Since back-extraction from an organic solvent into acid is an essential step for sample cleanup prior to electron-capture GLC analysis (23), it was necessary to convert them to their respective stable *o*-aminobenzophenones (24) by acid hydrolysis. The benzophenones of clonazepam (J) and of flunitrazepam (V) are eluted as well-resolved symmetrical peaks, using the polar phase OV-17 in the clonazepam assay and OV-225 in the flunitrazepam assay. Both compounds are quantitated in the nanogram range using the ^{63}Ni electron-capture detector.

This report describes an assay procedure for clonazepam which is also applicable with minor modifications for the determination of flunitrazepam (I), its *N*-desmethyl metabolite (II), and nitrazepam (25). The method described for the determination of clonazepam uses flunitrazepam as an internal reference standard and vice versa. Alternatively, nitrazepam can be substituted for flunitrazepam as the reference standard in the clonazepam assay, since its benzophenone (H) (Scheme I) has about the same retention time as that of V and is, therefore, resolved from J derived from clonazepam.

Studies on the biotransformation of nitrazepam (26, 27) and of clonazepam in man (28) have shown that the major pathway of elimination is by the reduction of the nitro group to the amine, which is then acetylated to the acetamide. Hydroxylation at the C-3 position also occurs, resulting in the elimination of these metabolites as their glucuronide and/or sulfate conjugates. Flunitrazepam is rapidly *N*-demethylated to yield II, the major blood metabolite in the dog, which is further metabolized by pathways analogous to that of clonazepam[3]. The 7-nitro-3-hydroxy analogs of clonazepam and flunitrazepam, which are also hydrolyzed to the same benzophenones (Scheme I), are removed by selective solvent extraction prior to acid hydrolysis to ensure the

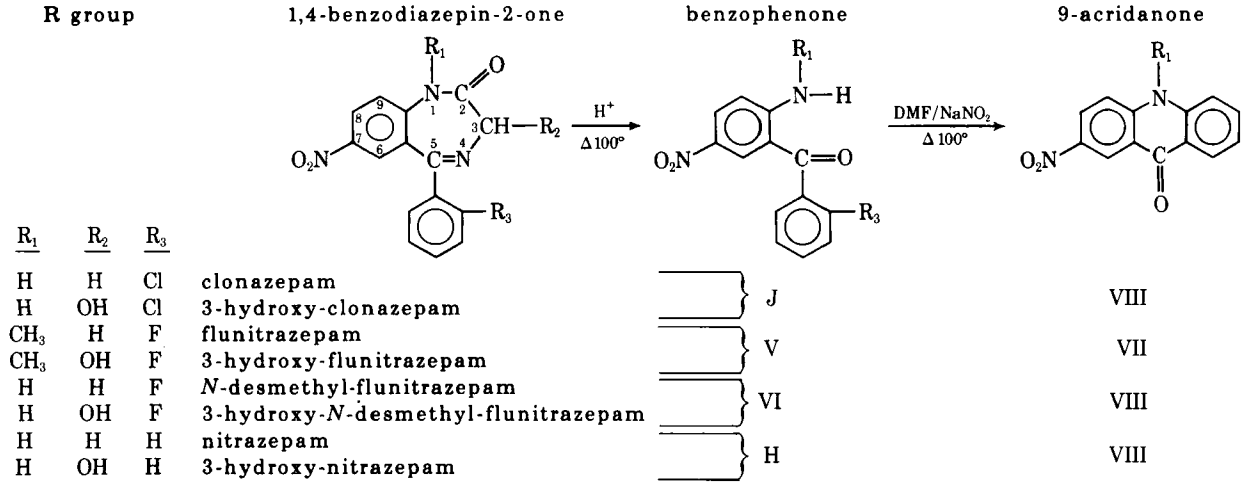
[1] Preferred USAN chemical name is (*o*-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one.

[2] Preferred USAN chemical name is 1,3-dihydro-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one.

[3] M. A. Schwartz and S. Kolis, unpublished data on file, Hoffmann-La Roche Inc., Nutley, NJ 07110, 1970.

Table I—Chemical Names and Physical Properties of Clonazepam, Nitrazepam, and Their Chemical Derivatives Referred to in Scheme I and Figs. 4 and 5

Compound	Chemical Name	Molecular Weight	Melting Point
A	7-Nitro-5-(2-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-(1*H*)-one (clonazepam)	315.70	238–240°
B	7-Nitro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-(1*H*)-one hemiacetonate	361.0	159–160°
C	7-Amino-5-(2-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-(1*H*)-one	285.74	230–232°
D	7-Amino-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-(1*H*)-one	301.74	340° dec.
E	7-Acetamido-5-(2-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-(1*H*)-one	322.77	292–300°
F	7-Acetamido-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-(1*H*)-one	343.77	187–190° dec.
G	7-Nitro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-(1*H*)-one (nitrazepam)	281.0	224–226°
H	2-Amino-5-nitrobenzophenone	242.0	157–159°
J	2-Amino-5-nitro-2′-chlorobenzophenone	276.7	118–120°
K	2,5-Diamino-2′-chlorobenzophenone	246.69	30–40°



Scheme I—Chemical reactions of clonazepam, flunitrazepam, nitrazepam, and their derivatives referred to in Tables I and II

specificity of the assay for the respective parent compounds.

The methods were applied to the determination of blood levels of clonazepam in man following single 2-mg oral doses (29, 30) and of flunitrazepam in the dog following single 2-mg/kg doses by intravenous and oral routes (29).

EXPERIMENTAL

Clonazepam (A) Column—The column packing[4] was a pretested preparation containing 3% OV-17 on 60–80-mesh Gas Chrom Q packed in a U-shaped, 1.21-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. This column effectively resolves the benzophenones J and V from coextracted impurities (Fig. 1) and has a useful lifespan of about 4–5 months of continuous use.

Flunitrazepam (I) Column—The column packing[4] was a pretested preparation containing 3% OV-225 on 60–80-mesh Gas Chrom Q packed in a U-shaped, 0.91-m (3-ft), 4-mm i.d. borosilicate glass column. This column was conditioned at 275 and 250° as described above. This column is preferred for the analysis of flunitrazepam (I) and its *N*-desmethyl metabolite (II), using clonazepam as the reference standard, since better resolution of their respective benzophenones V, VI, and J is achieved (Fig. 2), resulting in more precise quantitation.

Instrumental Conditions—The following conditions are common to both assays. A gas chromatograph[5] equipped with a ^{63}Ni electron-capture detector, containing a 15-mCi ^{63}Ni β-ionization source, was used. Argon–methane[6] (90:10) was used as the carrier gas, and the column head pressure was preset at 40 psig. The temperature settings were as follows: oven, 230°; injection port, 260°; and detector, 325°. The solid-state electrometer[7] 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 30 in./hr, and the time constant on the 1.0-mv recorder[8] was 1 sec (fsd). The response of the ^{63}Ni electron-capture detector (operated in the pulsed dc mode) to the benzophenones J, V, and VI showed maximum sensitivity at 60 v dc at a 300-μsec pulse rate and a 10-μsec pulse width. Under these conditions, 1.0 ng of each benzophenone (V, VI, and J) gave nearly full-scale pen response (>95%) on the 1.0-mv recorder.

In Clonazepam Assay—The flow rate used was adjusted to 120 ml/min (with the detector purge gas at 20 ml/min) to obtain retention times of 4.0 min for the reference benzophenone V and of 6.5 min for the benzophenone J derived from clonazepam (Fig. 1).

In Flunitrazepam Assay—The flow rate was adjusted to 100 ml/min (with the detector purge gas at 20 ml/min) to obtain re-

[4] Applied Sciences Labs., Inc., State College, Pa.

[5] Micro-Tek, model MT-220.
[6] Matheson, oil pumped and dry.
[7] Model 8169.
[8] Honeywell, model 194.

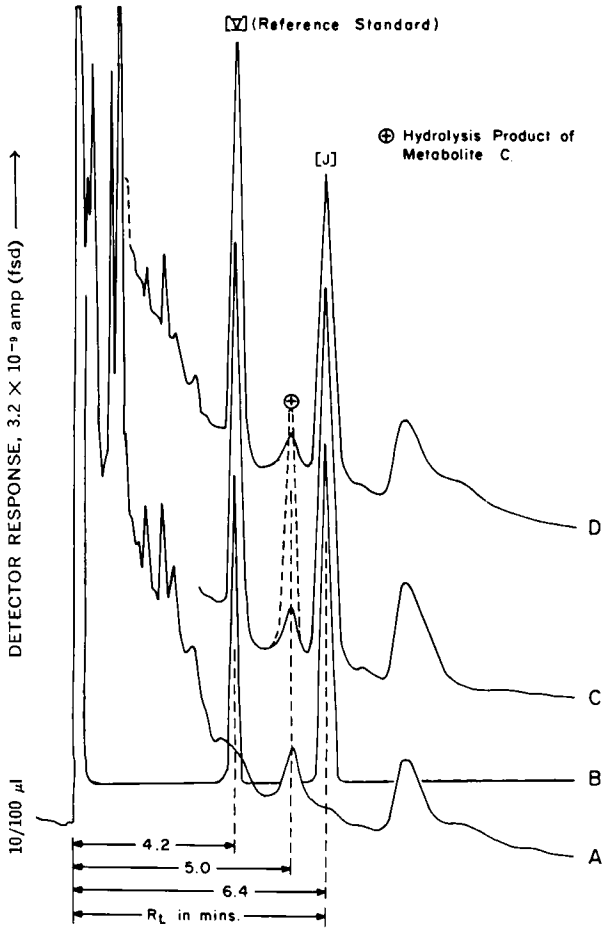


Figure 1—*Chromatograms of ether extracts of: (A) control blood, (B) authentic benzophenone standards, (C) control blood containing added clonazepam, and (D) patient blood 8 hr after 2-mg oral dose of clonazepam.*

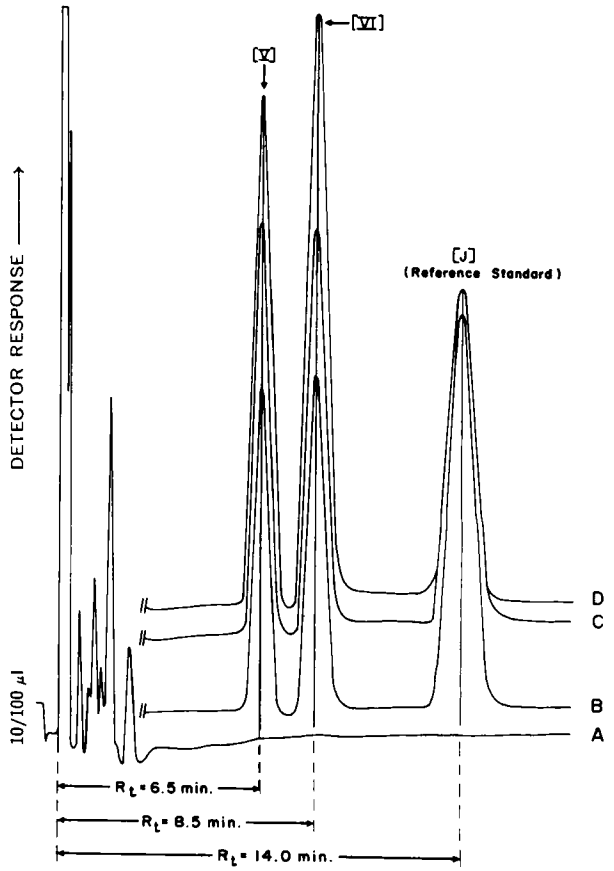


Figure 2—*Chromatograms of the ether extracts of: (A) control dog blood, (B) authentic benzophenone standards, (C) internal standards of I, II, and clonazepam (A) recovered from blood as the benzophenones after acid hydrolysis, and (D) dog blood after 2 mg/kg iv of flunitrazepam.*

tention times of 14 min for the reference benzophenone J and of 6.5 and 8.5 min for the benzophenones V and VI derived from flunitrazepam (I) and its *N*-desmethyl metabolite (II), respectively (Fig. 2). Under these conditions, the minimum detectable amount of clonazepam (A), flunitrazepam (I), and its *N*-desmethyl metabolite (II) is 0.5–1.0 ng of each/ml of blood or urine using a 2-ml specimen per analysis.

Preparation of Standard Solutions of Benzodiazepin-2-ones—The respective 7-nitro-1,4-benzodiazepin-2-ones [clonazepam (A), flunitrazepam (I), and its *N*-desmethyl metabolite (II)] that are required as internal analytical standards are listed in Tables I and II, respectively.

Weigh 10.0 mg each of clonazepam (A), flunitrazepam (I), and its *N*-desmethyl metabolite (II) (free base) into separate 10-ml volumetric flasks. Dissolve, using 1.0 ml of absolute ethanol followed by 1 ml of acetone, and then dilute to volume with 20% acetone–hexane. These stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions by suitable dilutions in 20% acetone–*n*-hexane, 100-µl aliquots of which are added to blood or urine as internal standards.

For the clonazepam assay, standard solutions containing 2.0–10.0 ng of clonazepam, each containing 10.0 ng of flunitrazepam (as the reference standard)/100 µl of 20% acetone–*n*-hexane solution, are prepared.

For the flunitrazepam assay, standard solutions containing 2.0–10.0 ng each of flunitrazepam and of its *N*-desmethyl metabolite (II), each containing 10.0 ng of clonazepam (as the reference standard)/100 µl of 20% acetone–*n*-hexane solution, are similarly prepared.

Benzophenone Standard Solutions for Electron-Capture GLC Analysis—The benzophenones J, V, and VI required as analytical standards are given in Tables I and II. Weigh 10.0 mg each of the free base of J, V, and VI into separate 10-ml volumetric flasks and prepare stock solutions analogous to those described previously for the intact benzodiazepin-2-ones. Prepare suitable dilutions of these stock solutions in 20% acetone–*n*-hexane, combining suitable aliquots of the respective dilutions to obtain the following working solutions.

For the clonazepam assay, prepare working solutions containing 2.0–10.0 ng of J, each containing 10.0 ng of V (the reference standard)/100 µl of 20% acetone–*n*-hexane.

For the flunitrazepam assay, similarly prepare working standard solutions containing 2.0–10.0 ng each of V and VI, each containing 10.0 ng of J (the reference standard)/100 µl of 20% acetone–*n*-hexane.

Ten-microliter aliquots of each solution (equivalent to 0.2–1.0 ng of compound) are injected into the gas chromatograph to establish the detector response and calibration linearity of the electron-capture GLC system for each day's analysis.

Reagents—All reagents were of analytical grade purity (>99%) and were used without further purification. The inorganic reagents were made up in double-distilled water and included: (*a*) 1.0 *M* (pH 9.0) H_3BO_3–Na_2CO_3–KCl buffer solution, prepared as described previously (23); (*b*) 4.0 *N* HCl–4.0 *N* H_2SO_4 (95:5 v/v) as the hydrolysis mixture, made fresh *daily* from the respective concentrated acids; and (*c*) 6.0 *N* NaOH.

The organic reagents used were: ether[9] (diethyl, absolute), analytical reagent grade, with a residue after evaporation and peroxide content of 0.0005% from a can opened no more than 3 days previously[10]; *n*-hexane[11], 99 mole % pure; acetone, reagent grade, and 20% acetone in *n*-hexane, both stored over anhydrous sodium

[9] Mallinckrodt.

[10] Check the purity of the ether by evaporating 20 ml to dryness, dissolve the residue in 100 µl of 20% acetone–*n*-hexane, and inject a 10-µl aliquot for electron-capture GLC. The chromatogram must be free of interfering peaks in the retention area of 2–15 min.

[11] Fisher H-301.

Table II—Chemical Names and Physical Properties of Flunitrazepam and Its Chemical Derivatives Referred to in Scheme I

Compound	Chemical Name	Molecular Weight	Melting Point
I	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (flunitrazepam)	313.29	170–171.5°
II	7-Nitro-5-(2-fluorophenyl)-3*H*-1,4-benzodiazepin-2(1*H*)-one	299.77	210–211°
III	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one	329.30	216–217°
IV	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one	315.28	193–195°
V	2-Methylamino-5-nitro-2′-fluorobenzophenone	274.26	186–187°
VI	2-Amino-5-nitro-2′-fluorobenzophenone	200.23	154–158°
VII	2-Nitro-10-methyl-9-acridanone	254.3	282–284°
VIII	2-Nitro-9-acridanone	240.2	>350°

sulfate; and bromthymol blue indicator solution [0.1% in ethanol–water (50:50)].

Extraction of Clonazepam—Into a 50-ml centrifuge tube containing 10.0 ng of flunitrazepam (reference standard), add 2.0 ml of blood (or 5 ml of urine), 5 ml pH 9.0 borate buffer, and 10 ml of ether. Seal the tube with a Teflon stopper, and extract by shaking for 10 min on a reciprocating shaker. Centrifuge the samples for 10 min at 2000 rpm (preferably in a refrigerated centrifuge at 0–4°). Repeat the extraction with another 10-ml portion of ether, centrifuge, and combine the ether extracts. Along with the samples, run a specimen of control blood or urine (taken from the subject prior to medication) and four 2-ml specimens of control blood (or 5-ml specimens of urine) containing 2.0, 4.0, 8.0, and 10.0 ng of clonazepam added as internal standards, with each tube containing 10.0 ng of flunitrazepam added as the reference standard. (The sensitivity limits of detection can be increased by extracting 4 ml of blood/assay. In this event, add 6 ml of pH 9.0 buffer and extract twice with 15-ml portions of ether. Combine the ether extracts and back-extract into 5.0 ml of the hydrolysis mixture. Wash the acid layer three or four times with fresh 10-ml portions of ether to effect the complete removal of the heavy layer of lipid material present at the interphase.)

Extraction of Flunitrazepam and Its *N*-Desmethyl Metabolite—Perform the extraction procedure as described for clonazepam, using 10.0 ng of clonazepam added as the reference standard into each unknown. Process along with a set of internal standards of 2.0, 4.0, 8.0, and 10.0 ng each of flunitrazepam (I) and its *N*-desmethyl metabolite (II) added to control blood or urine, with each tube containing 10.0 ng of clonazepam added as the reference standard.

The following steps are common to both assays. To the ether extracts from the respective assays, add 5.0 ml of the hydrolysis mixture containing 4 *N* HCl–4 *N* H_2SO_4 (95:5), shake for 10 min, and centrifuge for 5 min. Carefully remove the ether phase by aspiration without removing any of the acid (aqueous) phase[12]. Wash the acid extract twice with 15 ml of ether, shaking for 10 min and centrifuging for 5 min, and remove the ether by aspiration after each washing[12]. Hydrolyze the samples in a boiling water bath (100°) for 1 hr to convert the benzodiazepin-2-ones to their respective benzophenones (Scheme I).

Cool the samples in an ice bath, add 1 drop of bromthymol blue indicator, and carefully add 3.5 ml of 6.0 *N* NaOH dropwise until the blue end-point is reached. Extract this alkaline solution twice with 10 ml of ether by shaking for 10 min and centrifuging for 5 min. The ether extracts are combined sequentially in 15-ml conical centrifuge tubes and evaporated to dryness at 35–40° in the water bath of a rotary evaporator[13]. The residues are vacuum dried (under mild vacuum) for 15–30 min to remove all traces of moisture and dissolved in 100 μl of the 20% acetone–*n*-hexane mixture, a suitable aliquot of which (5–10 μl) is analyzed by electron-capture GLC. The benzophenone peaks due to their respective benzodiazepin-2-ones are identified by their respective retention times (Figs. 1 and 2), and their respective peak areas are determined by measuring peak height (centimeters) × width at half height (centimeters) using the slope baseline technique or by electronic digital integration.

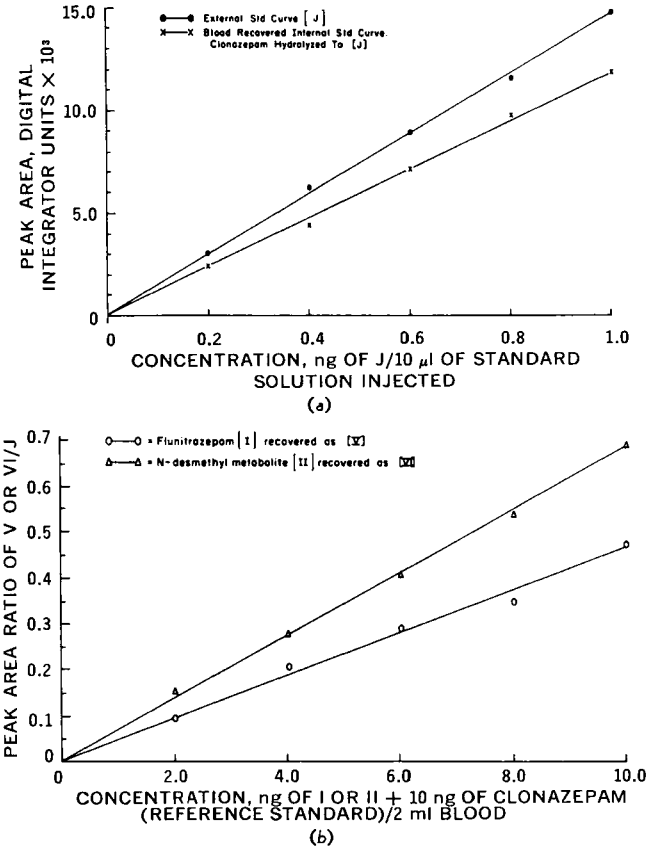


Figure 3—*Electron-capture detector calibration curves using:* (a) *the direct standard method (peak area* versus *concentration of the benzophenone (J) shown for clonazepam, and* (b) *the relative standard method (peak area ratio of V or VI/J* versus *concentration of I or II + 10 ng of clonazepam − reference standard)/2 ml of blood.*

Quantitation of Clonazepam (A), Flunitrazepam (I), and Its *N*-Desmethyl Metabolite (II) by Electron-Capture GLC Analysis—Calibration curves of the electron-capture detector response to the benzophenones J, V, and VI are made by plotting peak area *versus* concentration of the respective benzophenone, as shown for clonazepam in Fig. 3*a*. These calibration (external standard) curves are used not only to establish the linear response of the electron-capture detector and the stability of the GLC system but also to determine the percent recovery of internal standards. The internal standards are used to prepare calibration curves (Figs. 3*a* and 3*b*) for the quantitation of the concentration of clonazepam, flunitrazepam, and its *N*-desmethyl metabolite in biological specimens. These calibration curves have

[12] Save the ether phase if the determination of the 7-nitro-3-hydroxy metabolite is desired. Combine the ether washes with the original and evaporate to dryness. Dissolve the residue in 5 ml of the hydrolysis mixture, wash it twice with *n*-hexane as a cleanup step, hydrolyze, and process as for clonazepam.

[13] Buchler.

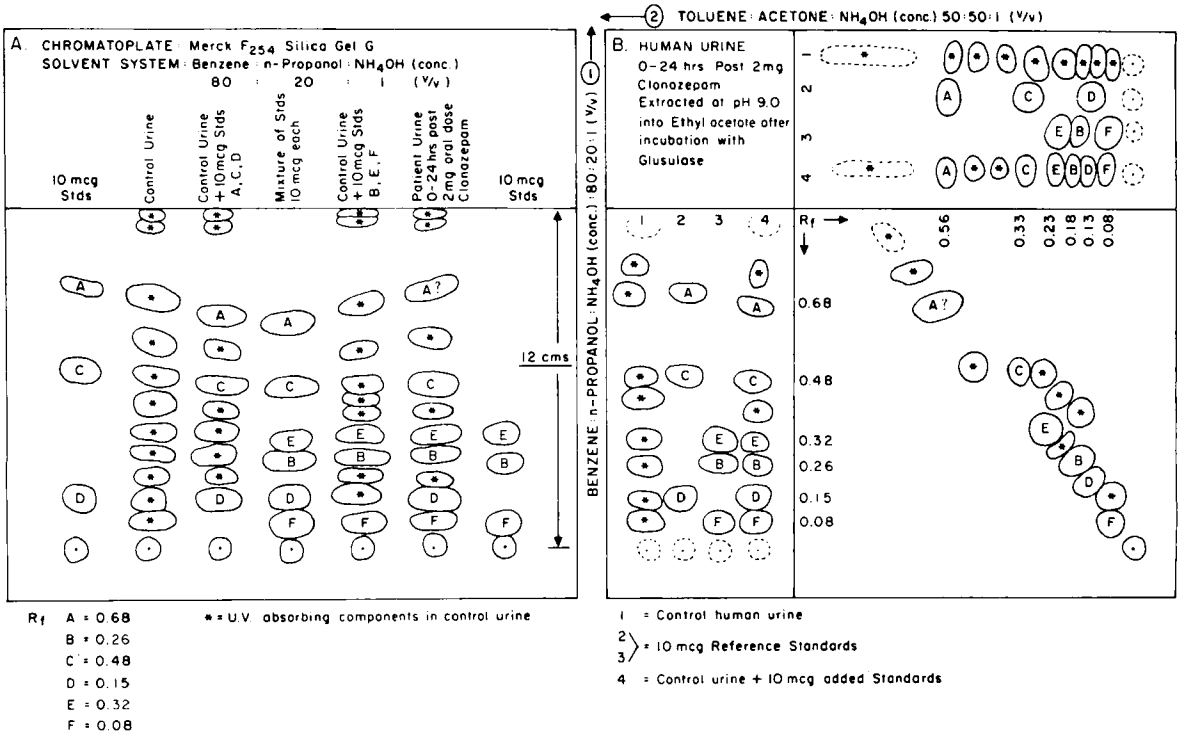


Figure 4—*Thin-layer chromatograms of human urine extracts containing clonazepam and its major metabolites developed by (A) one-dimensional and (B) two-dimensional chromatography.*

to be run each day with each set of unknowns and for each compound to be quantitated.

Calculations—Calibration curves using either the direct standard method (peak area *versus* concentration of benzophenone), as shown for clonazepam in Fig. 3*a*, or the relative calibration method (peak area ratio *versus* concentration), as shown for flunitrazepam and its metabolite (II) in Fig. 3*b*, can be used for the quantitation of either compound in the unknowns.

If the relative calibration (peak area ratio) method is used (Fig. 3*b*), the concentration in the unknowns is read directly (by interpolation) from the calibration curve because corrections for the aliquot of the total sample injected and the overall recovery of the compound measured are not required.

If the direct standard (peak area) method is used, then correction factors for the aliquot injected, the overall recovery of added standards, and the conversion of the benzophenone measured to its benzodiazepin-2-one equivalent have to be made.

The concentration of each component per milliliter of sample analyzed is calculated from the formula:

$$\frac{\text{ng of benzophenone found}}{\text{recovery factor of internal standards}} \times \frac{\text{dilution or aliquot factor}}{\text{ml of sample assayed}} \times \text{mol. wt.} \times \text{conversion factor}^{14} = \text{ng of clonazepam, flunitrazepam, or metabolite/ml of blood or urine} \quad \text{(Eq. 1)}$$

Determination of Percent Recovery—The overall recovery of clonazepam (A), flunitrazepam (I), and its *N*-desmethyl metabolite (II) was determined to be quantitative (*i.e.*, >85 ± 5.0%). When the relative calibration (peak area ratio) method (Fig. 3*b*) is used, the recovery factors of the respective compounds measured are assumed to remain constant. However, the percent recovery of added internal standards can be readily determined for either drug by using the direct calibration (peak area *versus* concentration) method (Fig. 3*a*).

Differential Pulse Polarographic Analysis of Major Urinary Metabolites of Clonazepam—The major clonazepam metabolites are the 7-amino (C), the 7-acetamide (E), and their respective 3-hydroxy analogs (D and F) (Table I) found mainly in the unconjugated form (28). Five milliliters of urine buffered to pH 9.0 is extracted with 2 × 15 ml of ethyl acetate. The residue of the combined extracts is separated by one-dimensional TLC in benzene–*n*-propanol–NH_4OH (concentrated) (80:20:1, no chamber saturation) (28) (Fig. 4A). The areas corresponding by R_f to metabolites C, D, E, and F are eluted with 2 × 5 ml of methanol, and its residue is dissolved in 4 ml of 0.1 *N* HCl and analyzed in a three-electrode cell (30) as previously described for other benzodiazepines (31). The peak produced by the reduction of the azomethine (>C_5=N_4—) group (common to all these metabolites) at around −0.600 v *versus* saturated calomel electrode (SCE) (Fig. 5) is used for their quantitation. The sensitivity limit of the assay is about 0.50–0.75 μg of each/5 ml of urine analyzed.

RESULTS AND DISCUSSION

Although electron-capture GLC methods for the determination of 1,4-benzodiazepin-2-ones as the intact compounds have been reported for medazepam, diazepam (23), and nitrazepam (32, 33), this principle was not applicable for clonazepam and flunitrazepam because of the poor electron-capture detector response of the former and the instability of the latter in acid. Extraction from blood into an organic solvent followed by back-extraction into acid and reextraction from an alkaline solution is essential for the necessary cleanup of the sample extract and for the quantitative recovery of basic 1,4-benzodiazepin-2-ones prior to electron-capture GLC analysis (23).

Therefore, it was necessary to convert them to a stable derivative, the benzophenone, by strong acid hydrolysis (Scheme I). Quantitative yields of the respective benzophenones were obtained by hydrolysis at 100° for 1 hr in a mixture of 4 *N* HCl–4 *N* H_2SO_4 (95:5 v/v). Although 4 or 6 *N* HCl also gave good yields of the benzophenones, this medium was not wholly suitable since erratic hydrolysis was obtained, resulting in a second product which affected the precision of the assay and overall recovery of the benzophenones of interest. Thus, in the case of clonazepam,

[14] Conversion factors: ng of J (benzophenone) × 1.14 = ng equivalent of clonazepam (A), ng of V × 1.14 = ng equivalent of flunitrazepam (I), and ng of VI × 1.15 = ng equivalent of *N*-desmethyl metabolite (II).

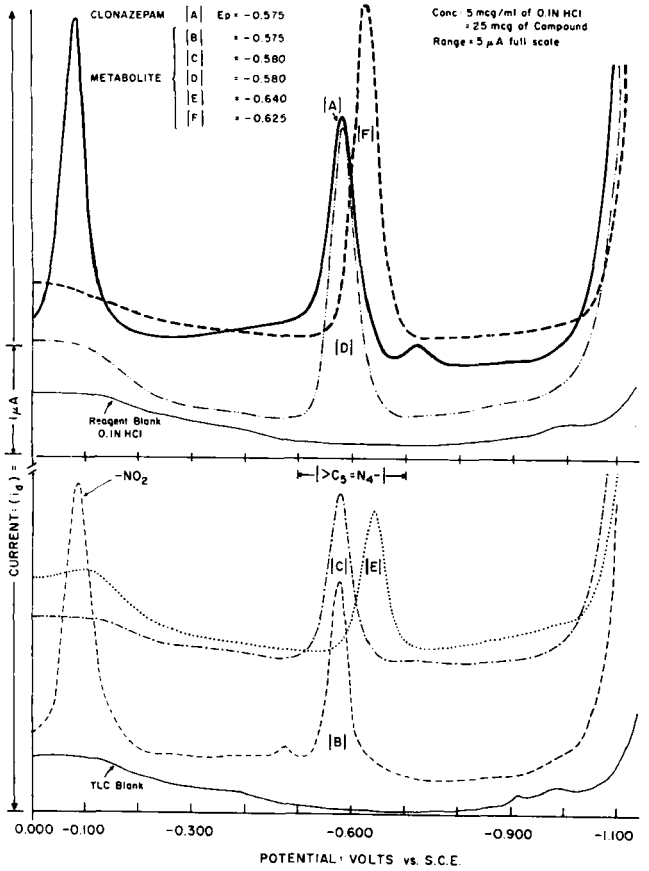


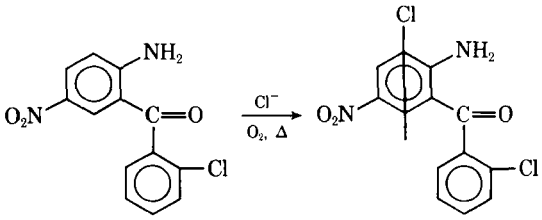
Figure 5—*Differential pulse polarograms of clonazepam and authentic standards of its major metabolites extracted from urine.*

the benzophenone J accounted for only 50–60% of the added parent compound, the losses being more acute in the lower nanogram concentration range.

The structure of this secondary product (which has a 1-min shorter retention time than J) was characterized by GLC–mass spectroscopy analysis to be a benzophenone compatible with the structure of a dichloro compound (Scheme II), which showed a molecular ion (*m/e*) at 310 amu, an increase of 34 amu over that of J (*m/e* 276 amu). This increase is compatible with the addition of a chlorine atom in the molecule.

This secondary product was more prevalent with hydrochloric acid solutions that had been standing for some time (>2–3 weeks). It may be due to an increase in the oxygen content on standing which, under the hydrolysis conditions, can either liberate or activate dissolved free chlorine in solution in the acid. The addition of the chlorine to the phenyl ring may be catalyzed by any one of the heavy metals present in trace amounts. The use of a freshly prepared mixture of 4 *N* HCl containing a small amount of 4 *N* H_2SO_4 (95:5) solved the problem and produced quantitative yields of the desired benzophenone (J). Similar problems encountered in the hydrolysis of flunitrazepam and its *N*-desmethyl metabolite to their respective benzophenones V and VI were overcome by the use of the same hydrolysis mixture. Following acid hydrolysis it is necessary to cool the sample in ice prior to neutralization with alkali to reduce the heat of neutralization evolved. This precaution is necessary because the benzophenones V and VI are readily cyclized at elevated temperatures in strongly basic media (especially in dimethylformamide containing sodium nitrite) to yield the 9-acridanone derivatives (34, 35) (Scheme I) due to the high reactivity of the fluorine group present in the 2′-position of the 5-phenyl ring. The benzophenone J does not readily cyclize to the acridanone, probably due to the greater chemical stability of the 2′-chloro group.

Assay Specificity—Since clonazepam and flunitrazepam are determined as their respective benzophenones J and V, any metabolite yielding the same benzophenone would interfere with the



Scheme II

specificity of the assay. Of the many possible metabolites, only the 7-nitro-3-hydroxy analogs (Scheme I) would yield the same benzophenones. Thus, for clonazepam, the presence of measurable amounts of Metabolite B would be a source of error. Although B is quantitatively extracted from blood at pH 9.0 into ether, it is only partially back-extracted into the hydrolysis mixture. The ether washes of the acid phase quantitatively remove any of Metabolite B present in the hydrolysis mixture prior to hydrolysis, leaving only the parent compound clonazepam (A) in the acid phase. This anomalous behavior of the 7-nitro-3-hydroxy analogs indicates that they are less basic than their parent compounds.

These ether washes are combined with the original blood ether extract (remaining after back-extraction of clonazepam into the acid hydrolysis mixture) for the subsequent determination of the 7-nitro-3-hydroxy metabolite (B) (see Footnote 12). Thus, the respective parent drugs clonazepam and flunitrazepam are the only compounds present in the hydrolysis mixture that would yield either J or V, therefore ensuring the specificity of the assay.

The 7-amino metabolite (C), if present, is also quantitatively extracted into ether from blood buffered to pH 9.0 but, on hydrolysis, it yields a product which is not the expected 2,5-diamino-2′-chlorobenzophenone (Compound K in Table I). Compound K has a retention time of about 4 min, whereas the derivative formed has a longer retention time of about 5 min. The electron-capture detector response to this product is very poor, and relatively high concentrations of the parent compound (C) are required (>200 ng) to produce a measurable peak of this derivative. Since it is resolved from the benzophenone peaks V and J, its presence even in trace amounts will not interfere with the quantitation of these benzophenones (Fig. 1). This product has not been chemically characterized as yet; hence, its overall recovery by electron-capture GLC and the efficiency of the hydrolysis of C have not been established. No measurable amounts of either Metabolite B or C have been noted in analyzed clinical blood specimens.

Recovery—The overall recovery of varying amounts of clonazepam (2.0–50 ng) added/ml of blood determined by electron-capture GLC and scintillation radiometry using ^{14}C-labeled clonazepam was 86 ± 6.5%. The recovery of the 7-nitro-3-hydroxy metabolite (B) determined by electron-capture GLC was about 70%. The overall recovery of flunitrazepam (I) and its *N*-desmethyl metabolite (II), added in varying amounts (50–250 ng/ml) to blood and determined by electron-capture GLC analysis[15], was 108.7 ± 8.0% for I and 110.6 ± 8.3% for II. This anomalous behavior is probably due to the formation of a Schiff-base "complex" with blood-extracted impurities, thereby reducing adsorption losses on the column during GLC analysis and resulting in an "apparent" enhanced recovery of compound. Since the quantitation of flunitrazepam and its metabolite (II) in the unknowns is based on their respective internal standard curves (Fig. 3*b*), the actual recovery factor is automatically compensated for yielding analytically valid data.

Differential Pulse Polarographic Assay for Urinary Metabolites of Clonazepam—Although spectrophotometric (26) and fluorometric (36) assays have been reported for the determination

[15] The development of the electron-capture GLC assay for flunitrazepam (I) and its *N*-desmethyl metabolite was originally done using an Aerograph model 205-B gas chromatograph with a ^{3}H-electron-capture detector of lower sensitivity, consequently requiring larger amounts (50–250 ng) of added standards. This instrument was adequate for measuring blood levels in animal studies at relatively high doses (2 mg/kg or greater) of administered flunitrazepam. Therapeutic doses in man are expected to be 2 mg or less and will require the use of the Micro Tek-220 chromatograph, with a 15-mCi ^{63}Ni electron-capture detector, or equivalent instrument.

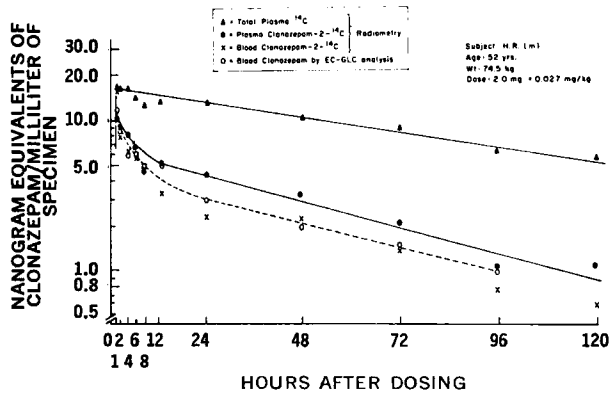


Figure 6—*Plasma levels of radioactivity and of intact clonazepam as a function of time following a 2-mg oral dose of micronized ^{14}C-clonazepam. Key: ▲, plasma total ^{14}C in nanogram equivalents of clonazepam per milliliter; ●, plasma ^{14}C-clonazepam in nanograms per milliliter; ×, blood ^{14}C-clonazepam; and ○, blood clonazepam determined by electron-capture GLC analysis in nanograms per milliliter.*

of nitrazepam and its major metabolites (which are analogous to those of clonazepam), they require chemical manipulations to produce either a suitable chromophore or a fluorophore for their determination. These assays also lack specificity, since all metabolites are quantitated as a total equivalent of the parent compound. The polarographic assay developed is specific by virtue of not only the chromatographic separation step (Fig. 4) but also of the specificity of the electrochemical reduction of the azomethine group, which produces a peak at about −0.600 v *versus* SCE (Fig. 5). Biological impurities present in the sample do not interfere with the reduction process, thereby resulting in a sensitive, specific, and precise quantitation.

The overall percent recoveries of clonazepam and its major metabolites added to urine and separated by TLC were: 88, A; 86, B; 68, C; 65, D; 93, E; and 88, F. The method was applied to the quantitation of urinary metabolites in man following a single 2-mg oral dose. Although Metabolites C, D, E, and F were detectable on the chromatoplate (under shortwave UV light), their concentrations following elution from the silica gel were at the sensitivity limit of the assay, *i.e.*, <0.5–0.75 μg/ml; hence, they are not reported. It is expected that the method will be useful in measuring urinary metabolites following chronic administration of clonazepam.

Application of the Electron-Capture GLC Method in Biological Specimens—The accuracy of the chemical assay was verified in one subject who received 2 mg of micronized labeled clonazepam-2-^{14}C administered in a gelatin capsule containing anhydrous lactose as a diluent. Duplicate whole blood specimens (10 ml, oxalated) were drawn at appropriate time intervals up to 120 hr. One set of specimens was centrifuged immediately after being drawn, and the separated plasma was analyzed for its ^{14}C-clonazepam and total ^{14}C content as previously described (37). The duplicate set was analyzed for its ^{14}C-clonazepam content following ether extraction by TLC separation, and scintillation radiometry of the separated drug[16], and direct electron-capture GLC analysis. The plasma levels of total ^{14}C and of extracted ^{14}C-clonazepam are shown in Fig. 6 and indicate measurable levels up to 120 hr postdosing. The half-life of elimination of the unchanged drug was calculated to be about 47 hr in this subject. The levels of total plasma ^{14}C indicate the presence of metabolites in plasma.

The blood levels of ^{14}C-clonazepam in this subject (Fig. 6) determined by both TLC radiometry and electron-capture GLC analysis showed good agreement, confirming the accuracy and sensitivity of the electron-capture GLC assay and its specificity for the intact drug.

Evidence that steady-state blood levels of clonazepam are achieved following chronic clonazepam therapy is presented in Table III. Blood levels monitored in a single subject administered 2 mg of clonazepam (tid) for the management of petit mal seizures showed that the blood levels of clonazepam ranged from 20 to 49 ng/ml over 4 months, suggesting steady state. No measurable blood levels of either the 7-nitro-3-hydroxy metabolite (B) or the 7-amino metabolite (C) were seen in this subject.

The electron-capture GLC assay was also used successfully for the determination of clonazepam blood levels in man following the administration of 2 mg of the micronized drug in a tablet formulation in studies on the blood level profile and the pharmacokinetics of clonazepam in man and of flunitrazepam in the dog (29).

[16] M. A. Schwartz, C. W. Williams, and A. S. Leon, unpublished data on file, Hoffmann-La Roche, Inc., Nutley, NJ 07110, 1972.

Table III—Blood Levels of Clonazepam in Human[a] following Chronic Administration

Date	Time Drawn	Blood Concentration, ng/ml
11/16/71	10:00 am	26.4
11/17/71	10:00 am	34.3
11/18/71	10:20 am	35.8
11/18/71	3:00 pm	27.2
11/18/71	1:00 pm	19.8
1/4/72	9:00 pm	49.6
1/5/72	12:30 pm	31.0
1/5/72	7:05 pm	21.7
3/9/72	9:00 pm	42.0
3/10/72	10:00 am	34.3
3/10/72	7:00 pm	25.6

[a] Subject: J. N., female, 51 kg. Dose: 2.0 mg tid taken at 7:00 am, 3:00 pm, and 9:00 pm. Total: 6 mg = 0.04 mg/kg. Data courtesy of Dr. H. J. Kupferberg, National Institute of Narcotics and Dangerous Substances, National Institutes of Health, Bethesda, Md.

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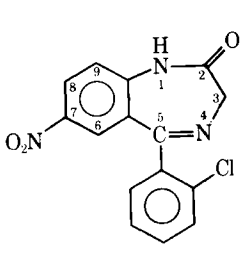
Pharmacokinetic Profiles of Clonazepam in Dog and Humans and of Flunitrazepam in Dog

S. A. KAPLAN[x], K. ALEXANDER, M. L. JACK, C. V. PUGLISI, J. A. F. de SILVA, T. L. LEE, and R. E. WEINFELD

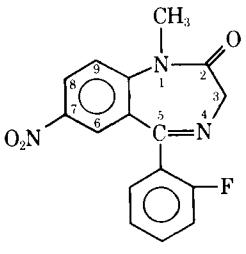
Abstract □ Clonazepam appears to be well absorbed, exhibiting peak blood levels of 6.5–13.5 ng/ml at 1–2 hr after administration in human subjects receiving 2-mg single oral doses. The "apparent" half-lives of elimination ranged from 18.7 to 39 hr in the subjects studied. Less than 0.5% of the dose is recoverable in the urine, suggesting complete biotransformation and/or alternative routes of excretion. Flunitrazepam in the dog is rapidly eliminated and exhibits a "first-pass" metabolism effect following oral administration, whereby the *N*-desmethyl metabolite is the major detectable drug component in the blood. Neither the drug nor its *N*-desmethyl metabolite is detected in the urine, suggesting extensive and complete biotransformation.

Keyphrases □ Clonazepam—blood levels and urinary excretion in dogs and humans □ Flunitrazepam—blood levels and urinary excretion in dogs □ Pharmacokinetic profiles—clonazepam in dogs and humans, flunitrazepam in dogs

The 7-nitro-1,4-benzodiazepin-2-ones, clonazepam and flunitrazepam, have shown anticonvulsant properties in several animal species (1–5). In addition, clonazepam is clinically effective in controlling minor motor seizures (petit mal) at minimal effective doses in the range of 1–2 mg/day orally (6–9). Metabolic



clonazepam



flunitrazepam

and pharmacokinetic studies of clonazepam in the rat, dog, and humans were previously reported (10). Flunitrazepam is a clinically effective hypnotic (11, 12) and an anesthesia induction agent (13, 14).

The present study reports the pharmacokinetic profile of clonazepam in the dog and humans and of flunitrazepam in the dog based on the evaluation of blood level and urinary excretion data determined using a sensitive and specific electron-capture GLC assay procedure (15). The assay follows for the measurement of clonazepam following its administration and of flunitrazepam and *N*-desmethyl flunitrazepam following flunitrazepam administration.