Journal of Chromatography, 99 (1974) 461–483

CHROM. 7648

DETERMINATION OF FLURAZEPAM (DALMANE®) AND ITS MAJOR METABOLITES IN BLOOD BY ELECTRON-CAPTURE GAS–LIQUID CHROMATOGRAPHY AND IN URINE BY DIFFERENTIAL PULSE POLAROGRAPHY

J. ARTHUR F. de SILVA, CARL V. PUGLISI, MARVIN A. BROOKS and MARTIN R. HACKMAN

Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, N.J. 07110 (U.S.A.)

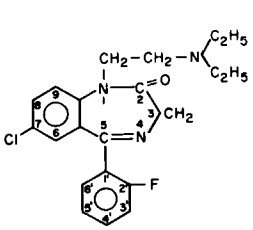
SUMMARY

A sensitive and specific electron-capture gas chromatographic (EC-GLC) assay was developed for the determination of flurazepam and its major blood metabolites with a sensitivity limit of 5–10 ng/ml of each compound. The major urinary metabolites are determined by differential pulse polarography. The EC–GLC assay was applied to the determination of blood levels in man following single and multiple 30-mg oral doses of Dalmane. The polarographic assay was used to quantitate the major urinary metabolites in several subjects who received a single 90-mg oral dose of the drug.

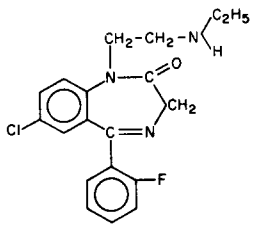
INTRODUCTION

Flurazepam*, 7-chloro-1-(2-diethylaminoethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one·dihydrochloride (I) was synthesized by Sternbach and co-workers[1,2], and is marketed as a hypnotic for the treatment of insomnia[3–10]. Studies on the biotransformation of flurazepam[11] showed that it was extensively metabolized in man and in the dog to the mono- (I-A) and didesethyl (I-B) metabolites and the hydroxyethyl (II), N-desalkyl (III) and the N-desalkyl-3-hydroxy (IV) metabolites. In addition, both compounds I and II were metabolized extensively in the dog to an acidic compound (V) by the oxidation of the alcohol side-chain to a carboxylic acid. The chemical structures of compounds I–V are presented in Fig. 1. The characterization of these metabolites and the synthesis of authentic reference compounds have been reported[12,13]. In man, the major blood component is III, whereas II (glucuronide conjugate) is the major urinary metabolite. Measurable amounts of I-A and I-B are also excreted, while I, III, IV and V are excreted in minor amounts[11,14].

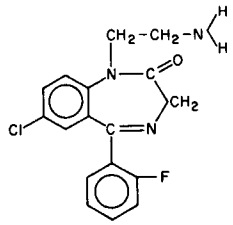
* Flurazepam·2HCl is the active drug substance in Dalmane® marketed by Hoffmann-La Roche Inc., Nutley, N.J., U.S.A.



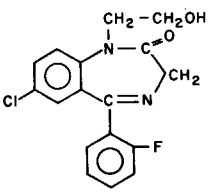
Flurazepam [I]



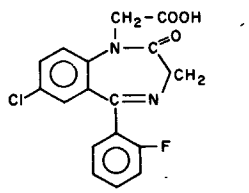
Mono-desethyl-flurazepam [I-A]



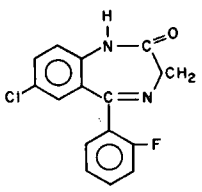
Di-desethyl-flurazepam [I-B]



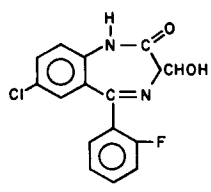
N-1-hydroxyethyl-flurazepam [II]



Flurazepam-N-1-yl-acetic acid [V]



N-1-desalkyl-flurazepam [III]



N-1-desalkyl-3-hydroxy-flurazepam [IV]

Fig. 1. Flurazepam and its major biotransformation products in man.

A spectrofluorimetric method[14] for the determination of blood levels of flurazepam and its major biotransformation products involves selective extraction of flurazepam (I) and its metabolites (II, III and IV) into diethyl ether from blood buffered to pH 9.0, back-extraction into 6 *N* hydrochloric acid; hydrolysis to their benzophenones followed by cyclization in dimethyl formamide–potassium carbonate to the highly fluorescent 9-acridanone derivatives. These derivatives are extracted into diethyl ether, separated by thin-layer chromatography (TLC) and their fluorescence determined in methanol–0.1 *N* hydrochloric acid (80:20). The sensitivity of the fluorimetric assay is of the order of 3–10 ng of each compound per milliliter of blood using a 4-ml specimen per analysis. The spectrofluorimetric assay was used to determine the blood-level profile of flurazepam and its major metabolites in man following chronic oral administration of 30 mg/day for 14 days[15]. The method was time consuming and not amenable to automation. Consequently, an electron-capture gas–liquid chromato-

graphic (EC-GLC) assay for the determination of flurazepam and its major blood metabolites was developed. The method is specific because it measures the intact 1,4-benzodiazepin-2-ones without derivatization. In addition, it lends itself readily to automation and is as sensitive as the fluorimetric assay. It is based on a modification of published procedures for other benzodiazepin-2-ones[16,17]. The EC-GLC assay employs a fluorosilicone liquid phase (QF-1), which completely resolves flurazepam from the metabolites (II, III and IV) present, thus permitting the specific and sensitive quantitation of these compounds utilizing the ^{63}Ni electron capture detector in the pulsed d.c. operational mode.

The EC-GLC assay was applied to the determination of blood levels in man following single and multiple 30-mg oral doses of Dalmane. The differential pulse polarographic assay was used to determine the urinary excretion in humans who received a single 90-mg oral dose of the drug.

GLC ANALYSIS OF FLURAZEPAM (I) AND ITS MAJOR METABOLITES IN BLOOD

Conditions for GLC analysis

*Column**. The column used was a U-shaped 5-ft., 4-mm I.D. silicanized borosilicate glass column containing 3% QF-1 on 60–80 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The column was conditioned at 265° for 4 h with "no flow" of carrier gas, followed by 12–16 h at 245° with carrier gas flowing at 40 ml/min. The useful life span of such a column was about 3–4 months of continuous use.

Instrumental parameters. A Micro-Tek gas chromatograph, Model MT-220, equipped with a 15-mCi ^{63}Ni electron-capture detector (Tracor Instruments, Austin, Texas, U.S.A.) was used. The carrier gas was argon–methane (90:10) (Matheson Gas Products, East Rutherford, N.J., U.S.A.) (oil pumped and dry), the column head pressure was 40 p.s.i.g., the flow-rate was 60 ml/min and the detector purge was 20 ml/min. The temperature settings were as follows: oven, 220°; injection port, 255°; detector, 310°. Under these conditions, the retention time of flurazepam (I) and its known blood metabolites (II, III and IV) are 11.2, 13.0, 7.7 and 4.0 min, respectively, whereas the reference standard diazepam (VI) has a retention time of 6.3 min. A typical chromatogram is shown in Fig. 2. The solid-state electrometer (Model 8169) response was $3.2 \cdot 10^{-9}$ A for full-scale deflection (f.s.d.) with the input set at 10^2 and the output attenuation at 32. The time constant on the 1.0-mV Honeywell recorder (Model 194) was 1 sec (f.s.d.), and the chart speed was 30 in./h. The response of the ^{63}Ni electron capture detector (operated in the pulsed d.c. mode) to flurazepam and diazepam showed maximum sensitivity at 60 V d.c. at a 270-μsec pulse rate and a 4-μsec pulse width. Under these conditions, 4 ng of flurazepam and 3 ng of diazepam per 10 μl injected give nearly full-scale pen response on the 1.0-mV recorder. The minimum detectable amount of flurazepam is 5–10 ng/ml in blood.

Calibration of flurazepam and its metabolites by GLC. A calibration (external standard) curve of either the peak area or the peak area ratio of flurazepam and the

* The empty column is deactivated by treating it with a 1% aqueous solution of Siliclad (Clay-Adams Co., New York, U.S.A.). Fill the column with the solution, let it stand at room temperature for 10 min, rinse with distilled water and heat in a conditioning oven at 125° for 35 min. The column is then filled with the support and conditioned as described.

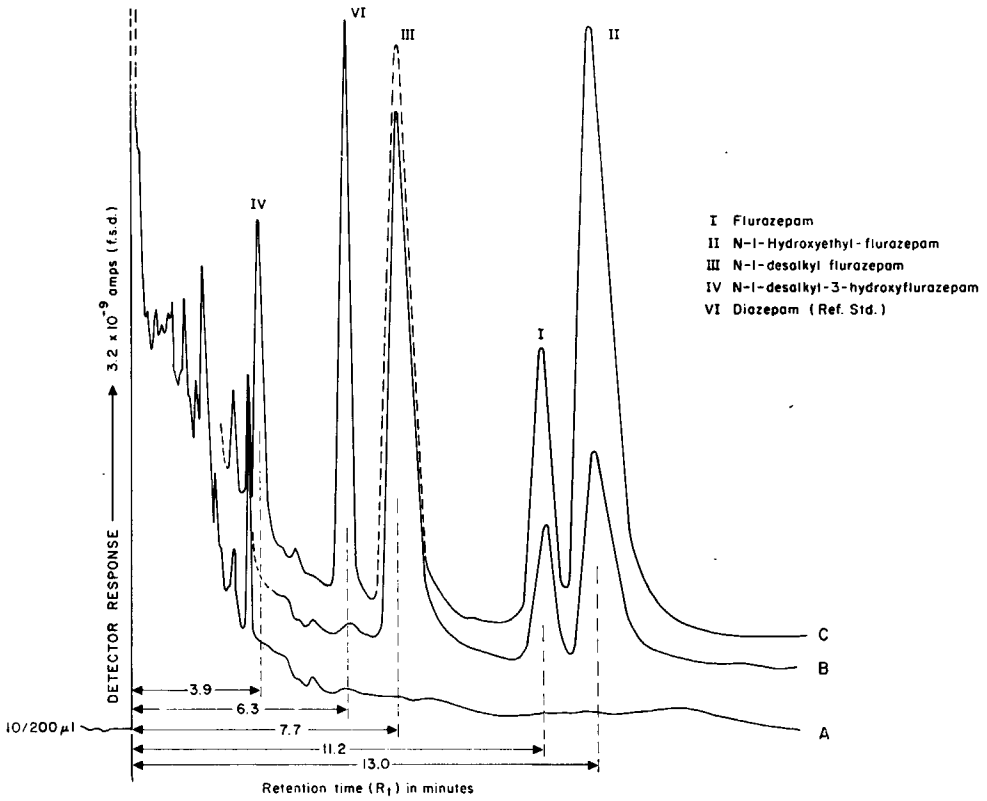


Fig. 2. Chromatograms of the EC-GLC analysis of blood diethyl ether extracts for therapeutic level analysis. (A) Control blood extract; (B) blood extract after 30-mg oral dose of flurazepam; (C) control blood extract containing added authentic standards.

metabolites II, III or IV to diazepam (reference standard) *versus* concentration of the 1,4-benzodiazepin-2-one per 10 µl of benzene–acetone–methanol (85:10:5) is constructed as shown in Fig. 3. A fresh calibration curve of the external reference standards and of the recovered internal standards is prepared for each day of analysis to establish the reproducibility of the GLC system. The non-zero intercept for II and IV is possibly due to adsorption on the column.

Assay in blood

Preparation of standard solutions. The respective authentic 1,4-benzodiazepin-2-ones that are required as analytical standards are listed in Table I. Weigh 10.00 mg each of the free base of flurazepam (I)*, its known blood metabolites (II, III and IV) and diazepam (VI) (reference standard for GLC analysis) into separate 10-ml volu-

* Weigh 11.90 mg of flurazepam·2HCl equivalent to 10.00 mg of free base, dissolve in water in a 50-ml centrifuge tube, add sufficient 0.1 *N* NaOH until the solution turns turbid, extract into 3 × 10 ml of diethyl ether, evaporate off the ether in a 10-ml tared volumetric flask and combine the extracts by successive evaporation. Vacuum dry to constant weight of the free base (oil). Dissolve in acetone and make up to volume as described.

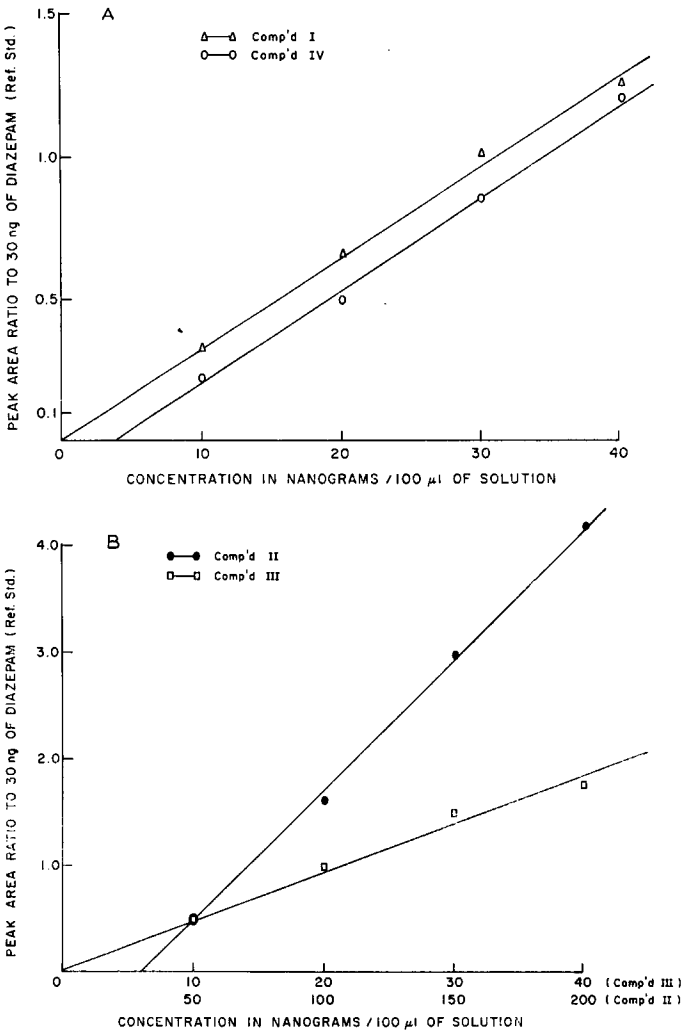


Fig. 3. External calibration curves for the EC-GLC analysis of flurazepam (I) and metabolites II, III and IV.

metric flasks, dissolve in 2.0 ml of acetone and make up to volume with benzene–acetone–methanol (85:10:5) to yield stock solutions (A) containing 1 mg/ml. Make serial dilutions of the respective solutions (A) and combine suitable aliquots of these dilutions to yield working solutions B_1 to B_4 containing the concentrations of each compound per 0.1 ml of benzene–acetone–methanol (85:10:5) indicated in Table II.

Aliquots of 10 μl of these solutions are injected as external standards for establishing the gas chromatographic parameters and optimizing the EC detector response to the compounds to be analyzed. Aliquots (100 μl) of the same solutions are added to blood as the internal standards for the calibration curve for the determination of the concentration in the unknowns and for the determination of percentage recovery (Fig. 3).

TABLE I

GENERIC AND SYSTEMATIC NOMENCLATURE OF THE 1,4-BENZODIAZEPIN-2-ONES REFERRED TO IN FIG. 1

Compound	*Generic name*	*Systematic name*	*Molecular weight*	*M.p. (°C)*
I	Flurazepam	7-Chloro-1-(2-diethylaminoethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one·2HCl	460.83	190–220 (decomp.)
I-A	Monodesethyl-flurazepam	7-Chloro-1-(2-ethylaminoethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one·2HCl	432.77	215–217
I-B	Didesethyl-flurazepam	7-Chloro-1-(2-aminoethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one·2HCl	404.7	221–223
II	N-1-hydroxyethyl-flurazepam	7-Chloro-1-(2-hydroxyethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one	332.76	110–115
III	N-1-desalkyl-flurazepam	7-Chloro-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one	288.5	205–206
IV	N-1-desalkyl-3-hydroxyflurazepam	7-Chloro-5-(2′-fluorophenyl)-3-hydroxy-1,3-dihydro-2H-1,4-benzodiazepin-2-one	304.72	195–198
V	Flurazepam-N-1-yl-acetic acid	2-[7-Chloro-5-(2′-fluorophenyl)-1,3-dihydro-2-oxo-2H-1,4-benzo-diazepin-1-yl]acetic acid	346.76	216–225 (decomp.)
VI	Diazepam	7-Chloro-5-phenyl-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (reference standard for EC-GLC analysis)	284.74	131–135

Reagents. All reagents must be of analytical-reagent grade (>99% purity) and all inorganic reagents were made up in distilled water. Diethyl ether (analytical-reagent grade diethyl ether (absolute) containing not more than 0.0005% of residue after evaporation and with a peroxide content of not more than 0.00005%; Mallinck-

TABLE II

COMPOSITION OF THE STANDARD SOLUTIONS

Standard solution	*ng of each per 0.1 ml solution*				
	I	*II*	*III*	*IV*	*VI**
B_1	10	50	10	10	30
B_2	20	100	20	20	30
B_3	30	150	30	30	30
B_4	40	200	40	40	30

* Diazepam is the reference standard for EC-GLC analysis.

rodt, St. Louis, Mo., U.S.A.) is used as the solvent for extraction. It must be used from a can either opened freshly or no more than three days previously*.

Other reagents are 1 *M* (pH 9.0) orthoboric acid–sodium carbonate–potassium chloride buffer (prepared as previously described[16]), benzene (nanograde) (Mallinckrodt), acetone (ACS reagent grade), methanol (absolute), 4.0 *N* hydrochloric acid and 4.0 *N* sodium hydroxide solution.

Extraction procedure for rapid toxicological analysis. Into a 50-ml centrifuge tube (PTFE-stoppered), add 1.0 ml of whole blood, 4 ml of pH 9.0, 1.0 *M* borate buffer and extract with 10 ml of benzene–methylene chloride (90:10) by shaking for 10 min on a reciprocating shaker. Along with the samples run a specimen of control blood (taken from the subject prior to medication or from a pooled control source) and four 1-ml specimens containing the residue of 100 μl of standard solutions B_1, B_2, B_3 and B_4. Centrifuge the samples at 5–10° for 10 min at 2400 rpm (1500 *g*) in a refrigerated centrifuge** and transfer an 8-ml aliquot into a 15-ml conical centrifuge tube. Evaporate to dryness at 60° in an N-EVAP (Organomation Associates, Worcester, Mass., U.S.A.) under a stream of clean, dry nitrogen. Vacuum dry the residues over Drierite pellets in a vacuum desiccator for 15 min to remove all traces of moisture, and dissolve the residues in 100 μl of benzene–acetone–methanol (85:10:5). Inject a 10-μl aliquot for EC-GLC analysis using the conditions previously described except that the oven temperature is lowered by 5° to 215° to improve the resolution of the peaks of interest from extraneous peaks due to endogenous impurities.

This direct procedure usually yields relatively "clean" chromatograms (Fig. 4) and is suitable for rapid routine toxicological analysis or following chronic drug administration where concentration is usually not a limiting factor. However, if greater sensitivity is required to monitor blood levels following therapeutic single doses, a larger sample volume (up to 4 ml of blood) has to be extracted. These larger samples require extensive clean-up to reduce the interfering impurities.

If the chromatograms contain too many interfering peaks so as to preclude accurate quantitation of the peaks of interest, add 8 ml of diethyl ether to the sample remaining after the above toxicological analysis, mix for 30 sec on a Vortex supermixer, add 5 ml of 4.0 *N* hydrochloric acid and proceed as described in the following therapeutic level analysis.

Extraction procedure for therapeutic level analysis. To a 50-ml centrifuge tube add 2.0 ml of blood, 5 ml of pH 9.0 borate buffer and 15 ml of diethyl ether for the first extraction. Along with the samples run a specimen of control blood (taken preferably from the patient prior to medication) and four 2-ml specimens of control blood containing the residue of 100 μl of B_1, B_2, B_3 and B_4 added as internal standards. Seal the tubes with a PTFE (No. 16) stopper, and extract by shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, Mich., U.S.A., at about 80–100 strokes/min). Centrifuge the samples at 0–4° in a refrigerated centrifuge for 10 min. Repeat the extraction procedure with a further 10-ml portion of diethyl ether, centrifuge, and combine the ether extracts. Add 5.0 ml of 4.0 *N* hydrochloric acid to the combined

* The chromatographic purity of each can should be checked prior to use by analyzing the residue of 20 ml evaporated to dryness, dissolved in 100 μl of benzene–acetone–methanol (85:10:5) and a 10-μl aliquot injected. The chromatogram should be free of any interfering peaks in the retention area of 2 min post-injection and beyond.

** A Model PR-J centrifuge with a No. 253 rotor (Damon/IEC, Needham, Mass., U.S.A.).

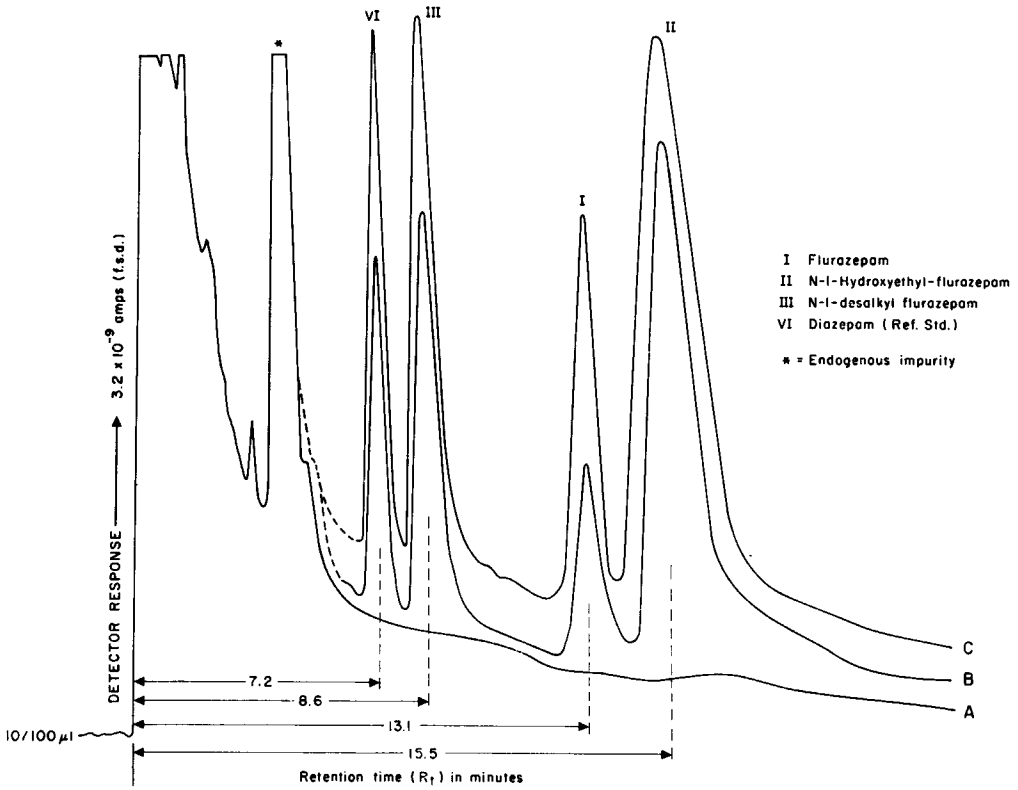


Fig. 4. Chromatograms of the EC-GLC analysis of blood benzene–methylene chloride (90:10) extracts for toxicological (overdosage) analysis. (A) Control blood extract; (B) control blood containing added authentic standards; (C) patient blood extract post dosage.

ether extracts, shake for 10 min and centrifuge for 5 min. Carefully remove the ether phase by aspiration without removing any of the acid (aqueous) phase. Wash the acid extract twice with 10 ml of diethyl ether, shaking for 10 min and centrifuging for 5 min, and remove the diethyl ether by aspiration after each washing. (The sensitivity limits of detection can be increased by extracting 4 ml of blood per assay. In this event, use 6 ml of pH 9.0 buffer and extract twice with 15-ml portions of diethyl ether, respectively. The ether extracts are combined as before and back-extracted with 5.0 ml of 4.0 *N* hydrochloric acid. The acid layer has to be washed 3–4 times with fresh 10-ml portions of diethyl ether in order to effect the complete removal of the heavy layer of lipid material which is present at the interphase.)

Cool the samples in an ice-bath and neutralize the acid by slowly adding 5.5 ml of 4.0 *N* sodium hydroxide solution. The final pH is about 10 (check pH with indicator paper). This alkaline solution is extracted twice with 10 ml of diethyl ether, shaking for 10 min and centrifuging for 5 min. The ether extracts are combined in a 15-ml centrifuge tube by serial evaporation to dryness at 35–40° in the water-bath of a Buchler rotary evaporator. The residues are vacuum dried for 30 min and dissolved in 100 µl of benzene–acetone–methanol (85:10:5), a suitable aliquot (10 µl) of which is injected for EC-GLC analysis. A typical chromatogram is shown in Fig. 2.

Calculations. The concentration of flurazepam and its metabolites in the unknowns is determined by interpolation from the calibration curves of the internal standards processed along with the unknowns, using either the direct calibration (peak area *versus* concentration) or the relative calibration (peak area ratio *versus* concentration) techniques. The percentage recovery of the internal standards of each compound is determined by comparing the slope value [peak area (cm^2) per nanogram of compound] of the internal standard curve with that of the respective external standard curve (Figs. 3A and 3B).

DIFFERENTIAL PULSE POLAROGRAPHIC ANALYSIS OF FLURAZEPAM (I) AND ITS MAJOR URINARY METABOLITES

Flurazepam undergoes extensive biotransformation in man to yield several metabolites[11], of which the hydroxyethyl analog (II) is the major urinary metabolite and is present mainly as a glucuronide and/or sulfate conjugate. The directly extractable (unconjugated) fraction in urine contains measurable amounts of metabolites I-A and I-B, while trace amounts of the parent drug and metabolites III, IV and V (Fig. 1) may also be present[11]. Owing to overlapping retention times (by EC-GLC) of flurazepam and I-A and of II and I-B, these urinary metabolites are more readily quantitated by differential pulse polarography (DPP) after thin-layer chromatographic (TLC) separation.

The urine specimen is first extracted at pH 11.0 with diethyl ether, which quantitatively removes I and any unconjugated I-A, I-B, II, III and IV, and is processed separately for TLC–DPP analysis. Metabolite V is not determined because it has to be extracted at acidic pH (5.5), resulting in too many interfering components on the TLC plate for identification. It can be quantitated by the differential extraction procedure previously published[14]. Quantitation of this minor metabolite was therefore omitted. The specimen is then titrated to pH 5.4, incubated with Glusulase enzyme (1% by volume) to deconjugate the major metabolite (II) and smaller amounts of IV and is then extracted into diethyl ether after adjusting the pH of the sample to 9.0. This extract is analyzed directly or following TLC separation by DPP. As metabolite II accounts for 22–46% of a given dose in the 0–24-h urine[11,14] its quantitation can be readily related to the dose administered and is therefore a reliable means of establishing the ingestion of flurazepam in cases of overdosage. Therefore, a direct assay for determining the amount of II conjugate by DPP analysis of 0.1 ml of urine diluted in a suitable buffer was also developed as a rapid diagnostic toxicological assay procedure.

Conditions for polarographic analysis

A Princeton Applied Research (PAR) Model 174 polarographic analyzer equipped with a PAR Model 172A drop timer (Princeton Applied Research Corp., Princeton, N.J., U.S.A.) was used in the differential pulse mode in conjunction with a three-electrode semimicro polarographic cell consisting of a dropping mercury electrode (DME), a saturated calomel electrode (SCE) and platinum wire as the auxiliary electrode, as previously described[18]. The pulse amplitude was −50 mV, the drop time was 1.0 sec and the drop rate was 2.42 mg/sec ($m^{2/3} \cdot t^{1/6} = 1.803$). The current range was set between 1.0 and 50 μA 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 −0.600 and −1.000 V *vs.* SCE and the polarograms were obtained on a Houston Omnigraph Model 2200-3-3 (X–Y) recorder (Houston Instruments, Bellaire, Texas, U.S.A.). The analytical peak due to the reduction of the azomethine ($>C_5=N_4-$) group, which is common to flurazepam and its metabolites, occurs between −0.780 and −0.815 V *vs.* SCE in pH 4 supporting electrolyte (Table III).

TABLE III

POLAROGRAPHIC DATA FOR FLURAZEPAM AND ITS METABOLITES

Polarographic parameters: mode, differential pulse; pulse, −50 mV; scan rate, 2 mV/sec; drop time, 1 sec; scan range, 1.5 V; reducible group, 4,5-azomethine. Supporting electrolyte: 1 *M* phosphate buffer, pH 4.0.

Compound	E_p *(V vs. SCE)*	*Sensitivity (μA/μg)*
I	−0.800	0.100
I-A	−0.780	0.092
I-B	−0.785	0.094
II	−0.815 (−0.955)*	0.076 (0.069)*
III	−0.785	0.063
IV	−0.830	0.126

* Supporting electrolyte: 1 *M* phosphate buffer, pH 7.0.

Assay in urine

Standard solutions. The analytical standards required are given in Table I. Dissolve the weight equivalent of 10.0 mg (free base) of each of compounds I-A, I-B and II in 10 ml of methanol to give stock solutions (A) each containing 1 mg/ml. Dilute 1 ml of each stock solution to 100 ml with methanol to give working solutions (B) containing 10.0 μg/ml. Fresh working solutions should be prepared every 2 weeks. Suitable aliquots of these solutions are added to urine as internal standards.

Reagents. In addition to the reagents used in the blood assay, the following are also required.

Phosphate (1.0 *M*) buffer (pH 11.0) is made by mixing 530 ml of 1 *M* dipotassium hydrogen orthophosphate and 470 ml of saturated trisodium orthophosphate. The pH is adjusted to 11.0 with 1 *M* potassium dihydrogen orthophosphate or saturated trisodium orthophosphate solution. The supporting electrolytes for DPP analysis, 1 *M* phosphate buffers of pH 4.0 and pH 7.0, are prepared as follows: phosphate buffer (pH 4.0) is prepared by mixing 840 ml of 1 *M* potassium dihydrogen orthophosphate and 160 ml of 1 *M* orthophosphoric acid and adjusting to pH 4.0 with 1 *M* potassium dihydrogen orthophosphate or 1 *M* orthophosphoric acid solution, whereas the pH 7.0 buffer is prepared by mixing 390 ml of 1 *M* potassium dihydrogen orthophosphate and 610 ml of 1 *M* dipotassium hydrogen orthophosphate; 1 *M* phosphate buffer (pH 5.4) is prepared by mixing 820 ml of 1 *M* potassium dihydrogen orthophosphate with 180 ml of 1 *M* dipotassium hydrogen orthophosphate and adjusting the mixture to pH 5.4 with 1 *M* potassium dihydrogen orthophosphate or 1 *M* dipotassium hydrogen orthophosphate solution. Other reagents included 0.5 *N* and 10 *N* sodium hydroxide solution, 2 *N* and 6 *N* hydrochloric acid, 95% methanol, ethyl acetate (Fisher Scientific, Pittsburgh, Pa., U.S.A.), glacial acetic acid and

ammonium hydroxide (J. T. Baker, Phillipsburgh, N.J., U.S.A.) and Glusulase enzyme preparation containing 100,000 units of glucuronidase and 50,000 units of sulfatase per milliliter (Endo Labs., Garden City, Long Island, N.Y., U.S.A.).

Rapid diagnostic toxicological assay for total benzodiazepines. To a 15-ml glass-stoppered conical centrifuge tube, add 100 μl of the urine sample. Along with the samples process a 100-μl specimen of control urine and separate 100-μl specimens of control urine containing 0.5, 1.0 and 2.0 μg of II added as internal standards (prepared by evaporating 0.05, 0.100 and 0.200 ml of the working standard (B) to dryness under nitrogen in separate 15-ml centrifuge tubes and adding 100 μl of control urine to the residues). To each tube add 1.90 ml of 1 *M* phosphate buffer (pH 7.0). Mix well on the Vortex supermixer and deoxygenate the samples for 5 min with nitrogen bubbled through a micro filter stick (SGA Scientific Inc., Bloomfield, N.J., U.S.A.; JD-5385-01 coarse porosity). The filter stick should be lightly sprayed with Anti-foam A spray (Dow Corning, Midland, Mich., U.S.A.) prior to deoxygenation of the samples in order to prevent excessive foaming. Transfer the deoxygenated sample into the polarographic cell and analyze the samples for total benzodiazepines by scanning between −0.800 and −1.100 V *vs.* SCE using the differential pulse mode of operation and the polarographic parameters described above. Measure the peak for total benzodiazepines at −0.955 V *vs.* SCE (Fig. 5). A typical calibration curve is shown in Fig. 6.

Specific assay for individual metabolites

(1) Determination of unconjugated compounds I-A and I-B in urine. To a 50-ml glass-stoppered centrifuge tube add 5 ml of urine sample, 0.5 ml of 0.5 *N* sodium hydroxide solution, 2 ml of 1 *M* phosphate buffer (pH 11.0) and 12.0 ml of diethyl ether. Stopper the tube with a PTFE stopper, shake on a reciprocating shaker for 10

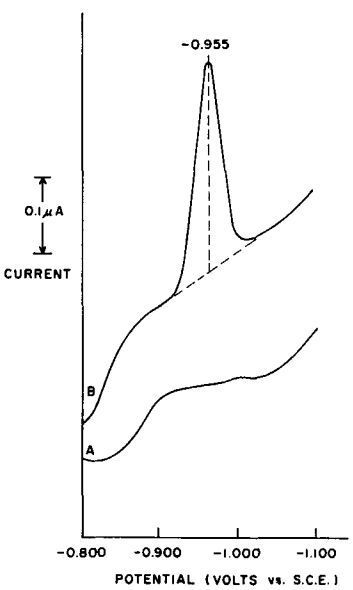


Fig. 5. Polarograms of direct analysis of 0.1 ml of urine diluted in pH 7.0 phosphate buffer as supporting electrolyte, for toxicological analysis. (A) Control urine; (B) patient urine specimen for total benzodiazepines.

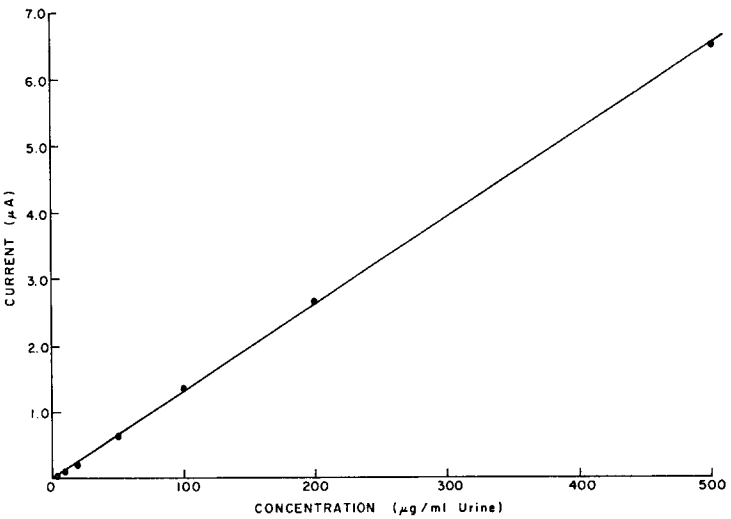


Fig. 6. Calibration curve for the DPP analysis of II added per millilitre of urine and analyzed directly for toxicological assay.

min at a moderate speed and then centrifuge for 5 min at 1500 *g*. Along with the samples process a 5-ml specimen of control urine and separate 5-ml specimen of control urine containing 2.5, 5.0 and 10.0 μg each of I-A and I-B as the internal standards (prepared by evaporating 0.25, 0.50 and 1.00 ml of the working standards (B) to dryness under nitrogen in separate 50-ml centrifuge tubes and adding 5 ml of control urine to the residues). (If the quantitation of compounds I–IV is desired, internal standard mixtures of each in the same concentrations as described for I-A and I-B are prepared. The TLC separation is modified to include an initial development in a vapor-saturated chamber of benzene–methanol–glacial acetic acid (90:10:10) until the solvent front has ascended 15 cm. The plate is air dried at room temperature to remove all traces of acetic acid and is re-developed in a vapor-saturated chamber of acetone–ammonium hydroxide (100:0.5). The R_F values of the respective compounds are given in Table IV.) Transfer a 10-ml aliquot of the diethyl ether extract into a 15-ml

TABLE IV

TLC SYSTEMS FOR THE SEPARATION OF FLURAZEPAM AND ITS METABOLITES USING QUANTUM (Q4F) SILICA GEL PLATES IN VAPOR-SATURATED CHAMBERS

Compound	R_F *in solvent system**		
	1	*2*	*3***
I	0.65	—	0.49
I-A	0.29	—	0.23
I-B	0.55	—	0.34
II	0.72	0.45	0.75
III	0.76	—	0.81
IV	0.63	0.66	0.66

* Developing solvent systems: (1) acetone–ammonia (100:0.5); (2) ethyl acetate–acetic acid (90:10); (3) benzene–methanol–acetic acid (90:10:10).

** First development in system 3 followed by second development in system 1.

conical glass-stoppered centrifuge tube and evaporate to dryness in a 45° water-bath (Evapomix, Buchler Instruments, Fort Lee, N.J., U.S.A.). Retain the extracted urine sample for the determination of conjugated II. Dissolve the residue in 100 μl of diethyl ether and transfer quantitatively on to a 20 × 20 cm Quantum Q4F silica gel TLC plate (Quantum Industries, Fairfield, N.J., U.S.A.). Rinse the tube with a 50-μl aliquot of diethyl ether and transfer to the TLC plate. Develop the plate in a vapor-saturated chamber using acetone–ammonium hydroxide (100:0.5) for 1 h to permit the saturation of the silica gel up to 15 cm. Air-dry the plate, examine it under short-wave UV light and locate the areas on the silica gel corresponding to I-A (R_F = 0.29) and I-B (R_F = 0.55) by reference to the R_F value of 10 μg of authentic standards run alongside the sample extracts. Scrape the silica gel areas from the plate and transfer them into 15-ml centrifuge tubes and add 5 ml of 95% methanol. Slurry the tubes on a Vortex supermixer for approximately 30 sec and centrifuge for 5 min at 1500 *g* to spin down the silica gel. Transfer the supernatant methanol into another 15-ml conical centrifuge tube and evaporate to dryness in a 65° water-bath under a stream of nitrogen.

Dissolve the residues individually in 2.0 ml of 1 *M* phosphate buffer (pH 4.0) just prior to DPP analysis. Mix well on the Vortex supermixer and deoxygenate the samples for 5 min with nitrogen bubbled in through a micro filter stick as before. Transfer the deoxygenated sample into the polarographic cell containing the three operational electrodes[18] and analyze the samples for I-A and I-B by scanning between −0.600 and −1.000 V *vs.* SCE using the differential pulse mode of operation and the polarographic parameters described above. The polarograms are recorded on an X–Y recorder and show analytical peaks at peak potentials (E_p) of −0.780 and

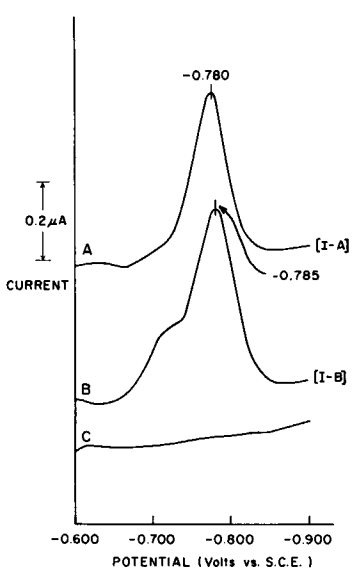


Fig. 7. Polarograms of authentic standards of (A) metabolite I-A, (B) metabolite I-B (showing the presence of the artifact) recovered from urine following TLC separation, and (C) control TLC blank.

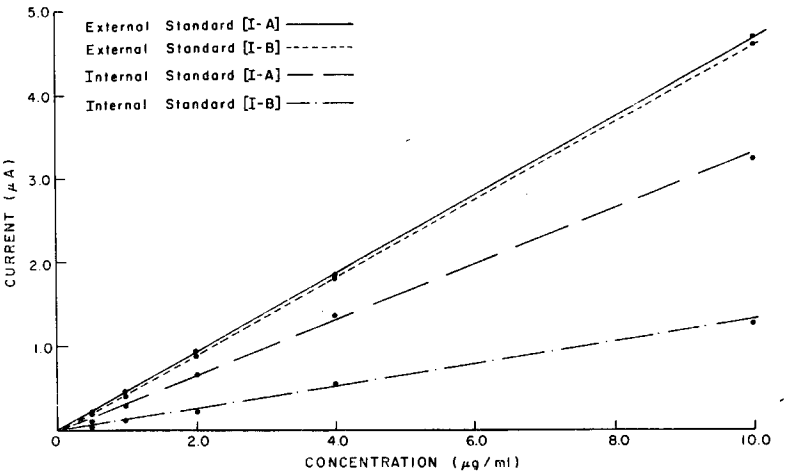


Fig. 8. Calibration curves for the DPP analysis of metabolites I-A and I-B as authentic (external) standards and as authentic (internal) standards extracted from urine and recovered following TLC separation.

—0.785 V *vs.* SCE for I-A and I-B, respectively (Fig. 7). Typical calibration curves are shown in Fig. 8.

(2) Determination of conjugated compound II in urine. The urine sample remaining from analysis (1) contains II and IV as glucuronide conjugates. Aspirate the samples to remove all residual traces of diethyl ether. Transfer the internal standard control urine specimen into fresh 50-ml centrifuge tubes containing 25, 50 and 100 μg of II, respectively (prepared by evaporating 0.025, 0.050 and 0.100 ml of stock solution A to dryness under nitrogen in separate 50-ml centrifuge tubes). Adjust the pH of the urine samples to pH 5.6 by adding 1.1 ml of 2 *N* hydrochloric acid and 3 ml of 1 *M* phosphate buffer (pH 5.4) and mix well on a supermixer. Add 0.25 ml of the Glusulase enzyme preparation to each tube and shake gently to produce a homogeneous mixture. Stopper the tube with cotton and place in a Dubnoff shaking incubator at 37° for 2.5 h. Cool the samples to room temperature and adjust the pH to exactly 9.0 using a pH meter. Titrate the sample with approximately 0.40 ml of 10 *N* sodium hydroxide solution. Add 12 ml of diethyl ether to the tube and stopper with a PTFE stopper, shake on a reciprocating shaker for 10 min at a moderate speed and then centrifuge for 5 min at 1500 *g*. Carefully transfer a 10-ml aliquot of the diethyl ether extract into a 15-ml conical glass-stoppered centrifuge tube. Evaporate the extracts to dryness in a 45° water-bath (Evapomix). (If the quantitation of metabolite IV is desired, then the residue is first separated by TLC on a Quantum Q4F silica gel plate in a vapor-saturated chamber containing ethyl acetate–glacial acetic acid (90:10).) Internal standards of compounds IV and II in the concentration ranges 2.5–10.0 and 25.0–100.0 μg per 5 ml of control urine, respectively, are processed as mixtures for the calculation of unknown samples. Compounds II and IV are identified by comparison with the R_F values of authentic standards (10 μg) of each run alongside the sample extracts, *viz.*, 0.45 and 0.66, respectively. (Because II is present in great excess over IV, this step can be omitted in the interests of expediency. Interference (if any) from IV in the quantitation of II is minimal.)

The residues are analyzed by DPP as described in assay (1). Compounds II and IV exhibit their peak potentials (E_p) at −0.815 and −0.830 V *vs.* SCE, respectively (Table III).

Calculations. The current (microamps) resulting from metabolites I-A, I-B and II and their overall recovery are determined as described previously[18–20], while the concentrations of these metabolites in the unknowns are determined by interpolation from their respective internal standard curves (Figs. 8 and 9), making the necessary corrections for the aliquots taken.

RESULTS AND DISCUSSION

Flurazepam undergoes extensive biotransformation in man, resulting in the presence of several metabolites (Fig. 1), measurable in both blood and urine[11,12]. This necessitates selective extraction and chromatographic separation prior to quantitation. The parent drug and the metabolites II, III and IV are all weakly basic compounds which can be quantitatively extracted into diethyl ether from biological materials buffered to pH 7.0 or 9.0. Metabolites I-A and I-B are more basic and require a pH $\geqslant$11 for extraction, while metabolite V is acidic and is extractable at pH values below 6.

The recovery of flurazepam and metabolites II–IV added to blood or urine buffered to pH 7.0 or 9.0 and extracted into diethyl ether has been determined by spectrophotometry as their benzophenones and by spectrofluorimetry as their 9-acridanone derivatives[14]. These compounds were shown to be quantitatively extracted above pH 7.0, but the diethyl ether extracts of blood and urine buffered to pH 9 with borate buffer gave cleaner control chromatograms on EC-GLC analysis and were preferred. Back-extraction of the compounds from the diethyl ether extract into acid

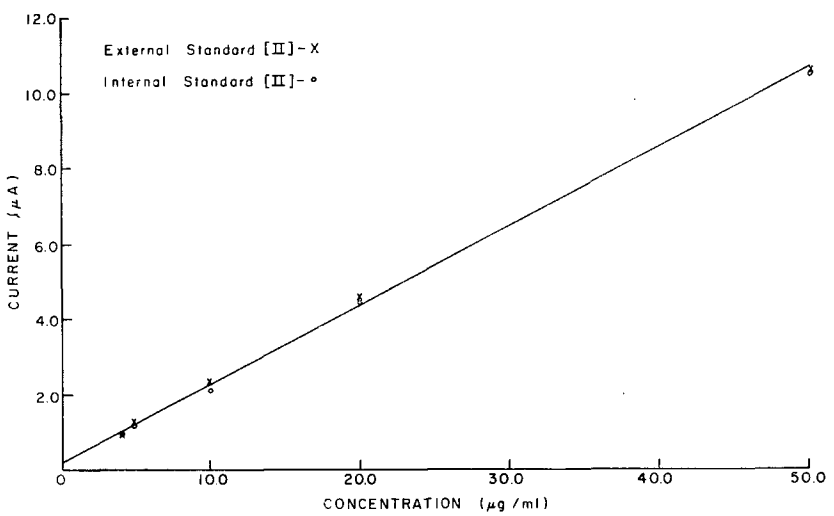


Fig. 9. Calibration curves for the DPP analysis of metabolite II as authentic (external) standards and as authentic (internal) standards extracted from urine and analyzed directly (without TLC separation).

(4 *N* hydrochloric acid), which is then washed with diethyl ether, is an essential clean-up step when analyzing more than 1 ml of blood to increase sensitivity. The compounds are re-extracted into diethyl ether following alkalinization and analyzed by EC-GLC. The chromatograms were devoid of interferences, permitting the quantitation of I, II, III and IV in blood (Fig. 2).

For toxicological analysis in cases of overdosage, where a rapid assay is essential, a modified extraction procedure is employed. The blood sample (1 ml or less) is buffered to pH 9.0; the drug and its major metabolites are extracted into benzene–methylene chloride (90:10). The extract is sufficiently clean for measuring the compounds of interest (Fig. 4), and sample dilution is often necessary to quantitate the peaks because they are off-scale. For therapeutic doses, the analysis of blood levels following a single 30-mg oral dose necessitates the extraction of up to 4 ml of blood and requires extensive clean-up of the sample, analogous to that required for medazepam or diazepam[16], in order to quantitate the compounds of interest (Fig. 2). Because flurazepam is rapidly and extensively metabolized, its levels in blood are usually low or non-measurable during the first hour. Consequently, extensive blood sampling should be undertaken during the first hour after dosing in order to be able to detect or measure the parent drug. In both therapeutic and toxicological situations, the major measurable blood component is the N-desalkyl metabolite (III), with lesser amounts of the hydroxyethyl metabolite (II) and trace amounts of flurazepam (I). The N-desalkyl-3-hydroxy metabolite (IV) was measured sporadically in blood only after chronic administration. Metabolites I-A, I-B and V are not measurable in blood either because they are not present in significant amounts and/or are also not recovered sufficiently at low concentration by the extraction procedures used. Their response to the EC detector is also very poor.

The chromatographic elution pattern of flurazepam and its known basic metabolites was determined under the experimental conditions described, using diazepam as the reference standard. The peaks were eluted in the following order of retention times: IV, 4.0 min; diazepam (VI), 6.3 min; III, 7.7 min; I-A, 10.8 min; I, 11.2 min; II, 13.0 min; and I-B, 13.5 min, indicating a potential interference from two compounds. However, as I-A and I-B are not recovered in the extraction step and their response to EC is very poor, they are eliminated as possible sources of interference in this blood assay. Consequently, only the quantitation of compounds I, II, III and IV is of significance in the blood assay. All of these compounds gave sharp symmetrical peaks on EC-GLC except II, which showed tailing due to the polarity of the hydroxyl group. However, both peak shape and sensitivity to EC-GLC can be greatly improved by preparing the trimethylsilyl (TMS) derivative with bistrimethylsilylacetamide (BSA) in pyridine. The resultant silyl ether was eluted as a sharp symmetrical peak with a 5-fold increase in sensitivity and a much shorter retention time, identical with that for III, the major blood metabolite. Owing to this interference, the derivatization of II was rendered impractical. As the peak shape of II and III improves significantly in the presence of blood-extracted impurities, they can be analyzed without derivatization and with the desired resolution.

Recovery and sensitivity limits of GLC assay

The overall recovery of flurazepam is $76\% \pm 6.0$ (S.D.) from blood. The recoveries of the major metabolites present in blood, hydroxyethylflurazepam (II),

N-desalkylflurazepam (III) and the N-desalkyl-3-hydroxy metabolite (IV) are 96% ± 8.0 (S.D.), 94% ± 6.0 (S.D.) and 42% ± 9.0 (S.D.), respectively. The limits of detection are of the order of 5–10 ng of flurazepam and III and 20–40 ng of II and IV per milliliter of blood using a 2-ml specimen per analysis. These limits can be increased by using a 4-ml specimen of blood per assay if needed.

The EC-GLC assay is preferred for the analysis of blood whereas the urinary metabolites are quantitated with better overall recovery using the DPP assay. This is particularly pertinent, as metabolites I-A and I-B are present in significant amounts in the urine in the directly extractable fraction and would therefore interfere in the quantitation of flurazepam and its hydroxyethyl metabolite (II) if analyzed by EC-GLC. Consequently, the urinary metabolites which are directly extractable (I, I-A, I-B and III (unconjugated)) and extractable after enzymatic deconjugation (II and IV) (glucuronide–sulfate conjugates) are best quantitated by selective extraction, TLC separation followed by DPP analysis of the separated components eluted from the silica gel.

DPP has been successfully applied to the determination of several types of drugs in their intact form, such as benzodiazepines[18,21], nitroimidazoles[22] and pyrimidine-containing compounds[20]. Others, such as glibornuride (a tolylsulfonylurea)[19], phenobarbital and diphenylhydantoin[23] were determined as their nitro derivatives. The 1,4-benzodiazepines can be quantitated in the submicrogram range due to the ease of reduction of the azomethine ($>C_5=N_4-$) group in dilute acids and the relatively high sensitivity ($\mu A/\mu g$) achieved. Consequently, flurazepam and its metabolites can be selectively extracted and quantitated with a minimum of clean-up following TLC separation and elution of the components because any co-extracted impurities do not interfere in the potential region of interest. The major components measured are directly extractable I-A and I-B and conjugated II (glucuronide conjugate), which is the predominant metabolite in urine. The concentrations of flurazepam (I) and metabolites III, IV and V are generally low and their total amount does not account for more than 1–2% of the dose. Hence their quantitation is not of significance relative to the amounts of metabolites I-A, I-B and II usually present. The DPP assay measures the intact underivatized benzodiazepin-2-ones and is specific by virtue of the TLC separation and the functional (azomethine) group reduced. The DPP assay can determine compounds I-A and I-B, which could not be previously determined by the spectrofluorimetric assay because of non-reproducible derivative formation. The toxicological assay described has the advantage that confirmation of the ingestion of flurazepam is possible within a few minutes employing only 0.1 ml of urine.

The following parameters were investigated in order to optimize the conditions for the polarographic analysis and elution efficiency of flurazepam and its metabolites. Previous polarographic studies on other benzodiazepines such as chlordiazepoxide[18] and clonazepam[24] indicated that the highest sensitivity ($\mu A/\mu g$) was obtained in dilute acids such as 0.1 *N* hydrochloric or sulfuric acid. Flurazepam and metabolites I-A and I-B, however, are unstable in dilute acids and are hydrolyzed to a benzophenone derivative by cleavage at the azomethine ($>C_5=N_4-$) bond, as shown for flurazepam in Fig. 10. The time course of the reaction in 0.1 *N* sulfuric acid can be readily followed owing to the fact that the two species present can be differentiated by the two peaks present, one at −0.595 V due to the reduction of the azomethine group of the

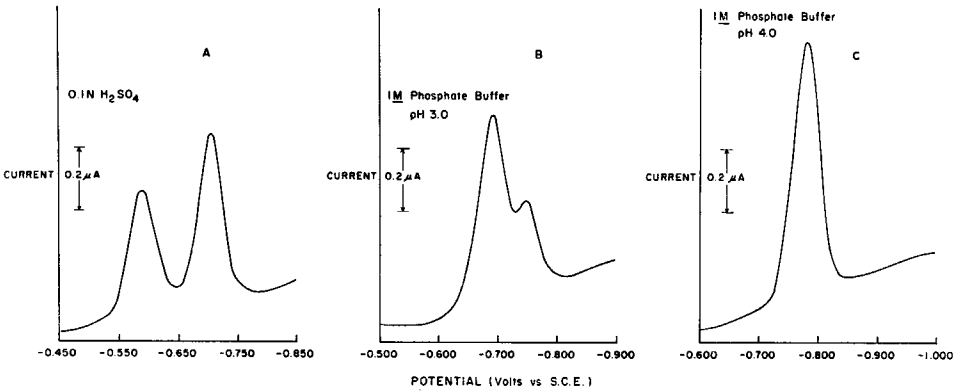


Fig. 10. Polarograms of flurazepam obtained in various supporting electrolytes. (A) 0.1 *N* H_2SO_4; (B) 1 *M* phosphate buffer (pH 3.0); (C) 1 *M* phosphate buffer (pH 4.0).

parent compound and the second at −0.715 V *vs.* SCE due to the reduction of the carbonyl group of the benzophenone (Fig. 10A). Increasing the pH towards neutrality led to the diminution of the benzophenone peak (pH 3.0), resulting in a single peak at pH 4.0 due only to the 4,5-azomethine (Figs. 10B and 10C).

The rate of hydrolysis of flurazepam was monitored in a solution containing 0.5 μg/ml of 0.1 *N* sulfuric acid by measuring the height of the reduction peaks for the 4,5-azomethine group of flurazepam and for the carbonyl group of its benzophenone derivative at −0.595 and −0.715 V *vs.* SCE, respectively (Fig. 11), and indicated a reaction half-life of 44.5 min. In analogous experiments, I-A and I-B showed reaction half-lives of 59.5 and 104.5 min, respectively. Compounds II, III, IV and V, however, are more stable in acids and show a major peak due to the 4,5-azomethine bond and

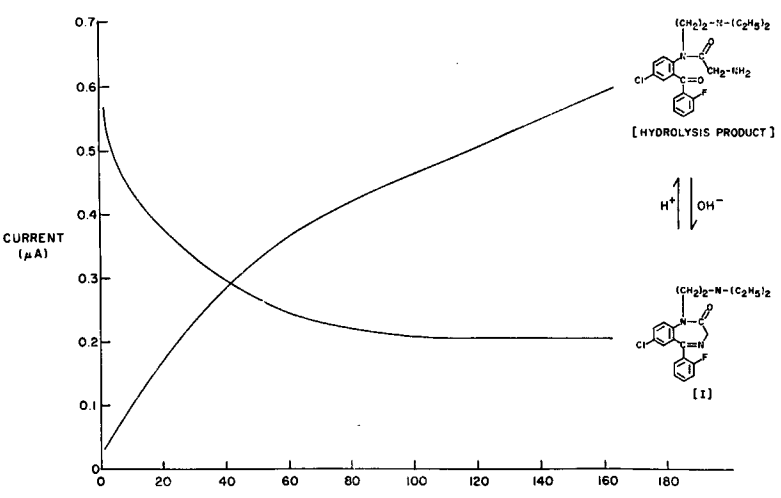


Fig. 11. Time course plot of the hydrolysis of flurazepam in 0.1 *N* H_2SO_4 determined by differential pulse polarography.

a minor peak due to the benzophenones. It was also noted that the reaction occurred in pH 3 buffer and that the carbonyl peak disappears with increasing pH of the supporting electrolyte, resulting in a single peak at pH 4.0 (Fig. 10C). Reversibility was obtained after the compound had remained in 0.1 *N* sulfuric acid for 1 h because adjustment of the pH to 7.0 resulted in only one peak due to the 4,5-azomethine group. This product, when extracted into diethyl ether and analyzed by TLC, showed only an R_F value identical with that of authentic flurazepam. The reversibility of the reaction to form the parent compound indicates that the benzophenone formed is an intermediate having the structure shown in Fig. 11. The ability of DPP analysis to differentiate between a mixture of a benzodiazepine and a benzophenone was used to analytical advantage in the determination of bromazepam [7-bromo-1,3-dihydro-5-pyridyl-1,4-benzodiazepin-2(1H)-one] and its major metabolites, 3-hydroxybromazepam and the benzoyl pyridines in urine[25]. The hydrolysis of flurazepam and its metabolites to the *o*-aminobenzophenones (an irreversible reaction) requires more drastic reaction conditions[14,26].

The choice of the optimal supporting electrolyte for polarographic analysis was determined using data relating the effect of pH upon peak potential (E_p) and current response ($\mu A/\mu g$) for each compound. Ten micrograms of each compound were determined in 0.1 *N* sulfuric acid, 1 *M* phosphate buffers ranging from pH 3 to 11, saturated trisodium orthophosphate (pH 12.8) and in 1.0 *N* sodium hydroxide solution (pH 14.0). The equation relating the change in peak potential (E_p) as a function of pH for I–IV is

$$E_p \approx -0.53 - 0.06\ \text{pH}$$

The data indicated that the highest reduction current ($\mu A/\mu g$), and hence sensitivity, was obtained at the lowest pH values. However, owing to the fact that compounds I, I-A and I-B give two peaks due to hydrolysis, acidic media (pH <3) are unsuitable for their quantitation.

Flurazepam and its metabolites gave a single reduction peak in pH 4.0 phosphate buffer due to the 4,5-azomethine group common to all of them; hence it was chosen as the supporting electrolyte for the quantitation of the individual metabolites with approximately equal sensitivity (Table V) for each compound. In the toxicological assay, the supporting electrolyte chosen was 1 *M* (pH 7.0) phosphate buffer because optimal resolution was obtained between the analytical peak (−0.955 V) and an interfering peak (−0.900 V) due to an endogenous impurity which limited the sensitivity of the assay to approximately 5 μg/ml (Fig. 5).

As TLC separation is an essential step for the specific quantitation of the individual metabolites, optimal resolution and elution recovery of the compounds of interest is critical. A previous study on the DPP analysis of chlordiazepoxide and its metabolites[18] demonstrated that optimal resolution of a number of benzodiazepines was obtained using commercially available silica gel plates (Quantum Industries Q4F), together with high elution recovery of these compounds into methanol. Although flurazepam and its metabolites were well resolved on these plates using the solvent systems given in Table IV, their elution recovery into methanol was low, particularly for I-A and I-B whose recoveries were of the order of 25–30%, indicating that methanol alone was not sufficiently polar to effect quantitative elution. Increasing the polarity

of methanol with mixtures of water in the range 0.1–5.0% indicated that a mixture of methanol and water (95:5) gave optimal elution recoveries of I, II, III and IV of about 85%, and of I-A and I-B of 65% and 75%, respectively.

Low elution recoveries of I-B resulted also from the formation of an artifact on TLC produced by dehydration of I-B. The identification of this artifact, 8-chloro-6-(2′-fluorophenyl)-1,2-dihydro-4H-imidazo-[1,2-*a*]-1,4-benzodiazepine (mol. wt. = 313.8; m.p. = 175–177°), was reported by Schwartz *et al.*[12]. Polarography of this compound in pH 4 phosphate buffer as the supporting electrolyte resulted in two peaks at −0.715 and −1.205 V *vs.* SCE, corresponding to the reduction of the 4,5-azomethine and the imidazolyl azomethine bonds, respectively. The presence of this artifact was noted in all the samples taken through TLC analysis by a shoulder at −0.715 V *vs.* SCE on the analytical peak measured at −0.780 V *vs.* SCE (Fig. 7B). This shoulder does not interfere with the quantitation of I-B.

Recovery and sensitivity limits of the polarographic assay

The overall recoveries of compounds I, II, III and IV (determined in the unconjugated fraction) in the concentration range 1–10 μg/ml in urine is of the order of 75% ± 5.0 (S.D.), whereas those of I-A and I-B are 71.2% ± 4.6 (S.D.) and 28.6% ± 2.7 (S.D.), respectively. The overall recovery of II (determined directly without TLC separation in the conjugated fraction) in the concentration range 4–50 μg/ml in urine is 95.1% ± 7.4 (S.D.).

The sensitivity limit is approximately 0.25–0.50 μg/ml for I-A, I-B and II using a 5-ml sample of urine per assay. This is based on the measurement of a peak 1.0 cm in height at a current range of 1.0 μA full-scale deflection. The sensitivity of the assay can be increased 3–4-fold by changing the drop time, scan rate and the operating current range on the polarograph.

Application of the assays to biological specimens

The blood-level profile of flurazepam and its major metabolites was determined in man following the administration of a single 30-mg oral dose and chronic administration of daily 30-mg oral doses for 14 consecutive days[15]. The spectrofluorimetric assay used in the above study[14] measured flurazepam (I) and its blood metabolites (II, III and IV) as their 9-acridanone derivatives. The assay was capable of resolving I, II and III, but was unable to resolve III from IV as they were measured as the same 9-acridanone derivative. The sensitivity limit of this assay (5–10 ng/ml) was increased to quantitate 0.5–1.0 ng/ml of I in blood using a 4-ml specimen per assay by direct (*in situ*) quantitation of the 9-acridanones on the TLC plate by spectrofluoridensitometry[27].

Determination of flurazepam and its metabolites in blood by EC-GLC. The EC-GLC assay was applied to the analysis of selected blood specimens remaining from the above pharmacokinetic study[15]. The data are given in Tables V and VI. Following a 30-mg single oral dose (subject 4, Table V), the parent drug was measurable between 1 and 6 h after dosing, whereas metabolite II was seen only at one point. The N-desalkyl metabolite (III) was measurable 1 h after dosing and was present in blood thereafter. On chronic administration, metabolite III was the major blood component, reached a steady state by the fourth day and declined gradually after medication was discontinued on day 14. The 3-hydroxy metabolite (IV) was measured sporadically in blood and did not exhibit any consistent blood-level profile.

TABLE V

BLOOD LEVELS OF FLURAZEPAM AND ITS MAJOR METABOLITE IN A MAN FOLLOWING THE ORAL ADMINISTRATION OF 30 mg OF DALMANE EVERY 24 h FOR 14 DAYS, WITH BLOOD LEVELS MONITORED UP TO 35 DAYS

Subject No. 4; weight 85.5 kg.

Day	*Hour*	*ng/ml of blood**	
		Flurazepam (I)	*N-Desalkyl-flurazepam (III)*
1	0	n.m.	n.m.
	1	5.0	3.1
	3	2.0	5.5
	6	1.2	5.0
	12	n.m.	6.4
	24	n.m.	8.6
2	24	n.m.	14.3
4	24	n.m.	19.0
8	24	n.m.	35.1
19	24	n.m.	27.0
21	24	n.m.	18.4
28	24	n.m.	8.5
35	24	n.m.	2.4

* n.m. = not measurable (<1.0 ng/ml in blood using a 4-ml specimen per assay).

TABLE VI

BLOOD LEVELS OF N-DESALKYLFLURAZEPAM (III) IN MAN FOLLOWING THE ORAL ADMINISTRATION OF 30 mg OF DALMANE EVERY 24 h FOR 14 DAYS, WITH BLOOD LEVELS MONITORED BETWEEN DAYS 14 AND 16 AFTER DRUG DISCONTINUATION

Subject	*Day*	*Hour*	*ng/ml of III in blood*
No. 2	14	1	32.5
(wt. 76.8 kg)		3	42.4
		6	30.4
		12	37.3
	15	24	30.0
	16	48	23.5
No. 3	14	1	41.0
(wt. 69.5 kg)		3	37.0
		6	35.5
		12	48.0
	15	24	30.0
	16	48	21.0

The blood-level data (Table VI) were taken in two subjects on days 14–16 after the last 30-mg dose of chronic administration. In these two subjects, metabolite III was the only measurable component present, the concentrations declined progressively with time from their respective steady-state levels, again confirming the findings of the previous pharmacokinetic study[15].

Determination of the major urinary metabolites by differential pulse polarography. The urinary excretion of flurazepam and its metabolites in humans who re-

ceived a single 90-mg oral dose of the drug was reported in a previous study[14]. Pooled specimens of 0–24 h post-dosing urine aliquots remaining from the above study were re-analyzed by DPP using the direct assay for total benzodiazepines and after TLC separation of the individual components. The data given in Table VII are in agreement with the previously reported spectrophotometric data[14] and with data using ^{14}C-labeled flurazepam[11]. In all three subjects, the excretion of flurazepam and metabolites II, III and IV in the unconjugated fraction and of IV as a conjugate accounted for a total of about 0.5% of dose. Metabolites I-A and I-B were the major components in the unconjugated form and accounted for 4–15% of the administered dose, while II conjugate was the major urinary metabolite in all three and accounted for about 30% of the dose in each subject. These findings are in better agreement with the ^{14}C-data[11] than with the spectrophotometric data[14]. Metabolites I-A and I-B were not determined by spectrophotometry[14] due to poor recovery and hydrolysis to the benzophenones under the conditions used in that assay, while the values for II were somewhat higher than those determined by DPP, probably due to interferences which were co-extracted with the benzophenone of II, which was the derivative used for quantitation.

TABLE VII

URINARY EXCRETION DATA ON FLURAZEPAM AND ITS MAJOR METABOLITES IN HUMANS WHO RECEIVED SINGLE ORAL 90-mg DOSES OF DALMANE

The sum of the amounts of flurazepam (I) and metabolites II, III and IV in the directly extractable (unconjugated) fractions yielded a total of 0.44, 0.36 and 0.54% for subjects 1, 2 and 3, respectively.

Subject	*Excretion period (h)*	*Form excreted*	*Compound measured*	*Concentration (μg/ml)*	*Milligram equivalent as flurazepam*	*Percentage of dose excreted*	*Direct assay for total benzodiazepines (%)*
1	0–24	Free	I-A	0.17	0.35	0.49	
			I-B	1.14	2.75	3.63	
		Bound	II	9.30	22.5	29.70	
						Total 33.82	42.2
2	0–24	Free	I-A	1.83	2.26	2.98	
			I-B	6.57	8.78	11.6	
		Bound	II	18.1	24.3	32.10	
						Total 46.68	50.2
3	0–24	Free	I-A	2.14	1.73	2.28	
			I-B	11.4	9.95	13.1	
		Bound	II	25.8	22.6	29.80	
						Total 45.18	43.8

The values obtained by the toxicological assay for "total" benzodiazepines by the direct analysis of 0.1 ml of urine diluted in the supporting electrolyte are also in good agreement with the summation of the individual metabolites obtained by TLC–DPP analysis (Table VII). Because the glucuronide conjugate of II is the main urinary metabolite measured by the direct assay in addition to I-A and I-B, the concentration

of total benzodiazepines in the urine is obtained using a correction factor (1.58) for the molecular weight of the glucuronide of II. Therefore

$$\frac{\text{Mol. wt. of glucuronide conjugate of II}}{\text{Mol. wt. of II (analytical standard)}} = \frac{526.84}{332.76} = 1.58$$

Although this correction factor is used in the calculation, no assumptions can be made regarding the relative DPP sensitivity of the conjugated and non-conjugated species, although the authentic standards of I–IV have approximately equal sensitivity to DPP analysis. Therefore, the toxicological assay is used only for the rapid confirmation of drug ingestion in cases of overdosage and to obtain an approximate amount of the ingested dose.

REFERENCES

1 L. H. Sternbach, G. A. Archer, J. V. Earley, R. I. Fryer, E. Reeder, N. Wasyliw, L. O. Randall and R. Banziger, *J. Med. Chem.*, 8 (1965) 815.
2 R. I. Fryer and L. H. Sternbach, *U.S. Pat. 3,691,157*, Sept. 12, 1972.
3 P. Bielmann, G. Pinard and L. Tetreault, *Int. J. Clin. Pharmacol.*, 6 (1972) 13.
4 A. Kales, M. B. Scharf and T. L. Tan, *Pharmacology and the Future of Man, Proc. 5th Int. Congr. Pharmacol., San Francisco, 1972*, Vol. 4, Karger, Basle, 1973, pp. 108–123.
5 A. Kales and M. B. Scharf, in S. Garattini, E. Mussini and L. O. Randall (Editors), *The Benzodiazepines*, Raven Press, New York, 1973, pp. 577–598.
6 W. C. Dement, V. P. Zarcone, E. Hoddes, H. Smythe and M. Carskadon, in S. Garattini, E. Mussini and L. O. Randall (Editors), *The Benzodiazepines*, Raven Press, New York, 1973, pp. 599–611.
7 A. J. Bond and M. H. Lader, *Psychopharmacologia (Berlin)*, 32 (1973) 223.
8 E. Domenichini, A. Pagnin, M. Fischetti and E. Cereda, *Curr. Therap. Res.*, 15 (1973) 534.
9 J. D. Frost, J. R. G. Carrie, R. P. Borda and P. Kellaway, *Electroencephalog. Clin. Neurophysiol.*, 34 (1973) 171.
10 D. A. Swanson, *S. Afr. Med. J.*, 47 (1973) 1339.
11 M. A. Schwartz and E. Postma, *J. Pharm. Sci.*, 59 (1970) 1800.
12 M. A. Schwartz, F. M. Vane and E. Postma, *J. Med. Chem.*, 11 (1968) 770.
13 J. V. Earley, R. I. Fryer, D. Winter and L. H. Sternbach, *J. Med. Chem.*, 11 (1968) 774.
14 J. A. F. de Silva and N. Strojny, *J. Pharm. Sci.*, 60 (1971) 1303.
15 S. A. Kaplan, J. A. F. de Silva, M. L. Jack, K. Alexander, N. Strojny, R. E. Weinfeld, C. V. Puglisi and L. Weissman, *J. Pharm. Sci.*, 62 (1973) 1932.
16 J. A. F. de Silva and C. V. Puglisi, *Anal. Chem.*, 42 (1970) 1725.
17 J. A. F. de Silva, I. Bekersky and C. V. Puglisi, *J. Chromatogr. Sci.*, 11 (1973) 567.
18 M. R. Hackman, M. A. Brooks, J. A. F. de Silva and T. S. Ma, *Anal. Chem.*, 46 (1974) 1075.
19 J. A. F. de Silva and M. R. Hackman, *Anal. Chem.*, 44 (1972) 1145.
20 M. A. Brooks, J. A. F. de Silva and L. D'Arconte, *Anal. Chem.*, 45 (1973) 263.
21 M. A. Brooks, J. A. F. de Silva and M. R. Hackman, *Amer. Lab.*, 5, No. 9 (1973) 23.
22 J. A. F. de Silva, N. Munno and N. Strojny, *J. Pharm. Sci.*, 59 (1970) 201.
23 M. A. Brooks, J. A. F. de Silva and M. R. Hackman, *Anal. Chim. Acta*, 64 (1973) 165.
24 J. A. F. de Silva, C. V. Puglisi and N. Munno, *J. Pharm. Sci.*, 63 (1974) 520.
25 J. A. F. de Silva, I. Bekersky, M. A. Brooks, R. E. Weinfeld, W. Glover and C. V. Puglisi, *J. Pharm. Sci.*, 63 (1974) in press.
26 B. C. Rudy and B. Z. Senkowski, in K. Florey (Editor), *Analytical Profiles of Drug Substances*, Vol. 3, Academic Press, New York, 1974, pp. 307–331.
27 J. A. F. de Silva, I. Bekersky and C. V. Puglisi, *J. Pharm. Sci.*, 63 (1974) in press.