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DETERMINATION OF CLONAZEPAM AND FLUNITRAZEPAM IN BLOOD BY ELECTRON-CAPTURE GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

An electron-capture gas–liquid chromatographic assay was developed for the determination of clonazepam (as its methyl derivative) and of flunitrazepam and its N-desmethyl metabolite in blood. The method was used to measure blood levels in man following single oral 2-mg doses of clonazepam and flunitrazepam, and has a sensitivity limit of 0.5 to 1.0 ng of compound per milliliter of blood.

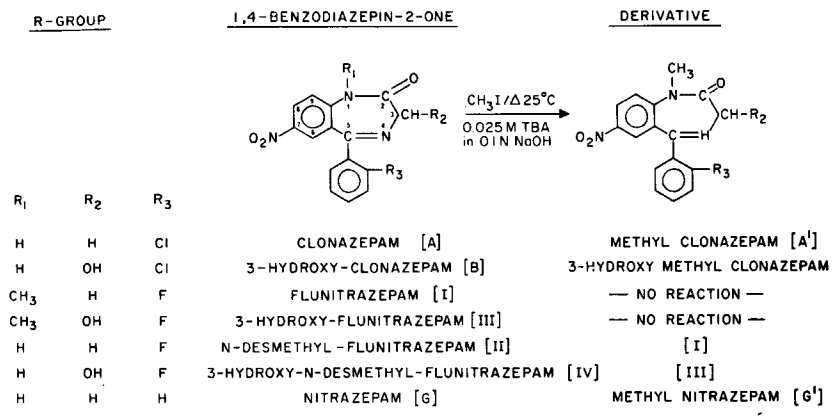
INTRODUCTION

The 1,4-benzodiazepine class of compounds[1] have yielded several clinically important drugs which are marketed as tranquilizers —Librium (chlordiazepoxide·HCl), Valium (diazepam), Nobrium (medazepam) and Serax (oxazepam)— and hypnotics —Mogadon (nitrazepam) and Dalmane (flurazepam·2 HCl).

The 7-nitro-1,4-benzodiazepin-2-ones[2], clonazepam (A) and flunitrazepam (I) (Fig. 1, Table I), have shown marked anticonvulsant and hypnotic properties in several animal species[3–6]. Clonazepam is clinically effective in controlling minor motor seizures (petit mal) at minimal effective doses in the range of 1 to 2 mg per day orally[7–10]. The biotransformation of clonazepam in the rat, the dog, and in man was reported[11] (Fig. 2). The major pathway is by the reduction of the $-NO_2$ group to the amine (C), which is then acetylated (E). Hydroxylation of each metabolite to the respective 3-hydroxy compounds B, D, and F is a minor pathway. Flunitrazepam is a clinically effective hypnotic[12–15] and an anaesthesia-inducing agent[16–20]. Flunitrazepam undergoes N-demethylation to yield II (Table I), which is further metabolized by pathways analogous to that of clonazepam*.

A sensitive and specific electron-capture gas–liquid chromatographic (EC-GLC) assay was reported[21] for the determination of clonazepam and flunitrazepam as their benzophenone derivatives produced by acid hydrolysis. The assay was used to measure blood levels of clonazepam in man following single 2-mg oral doses and of flunitrazepam in the dog[22]. This assay was time consuming for routine analysis of the large numbers

* M. A. Schwartz and S. Kolis, unpublished data on file (1970), Hoffmann-La Roche Inc., Nutley, N.J. 07110, U.S.A.



R_1	R_2	R_3	1,4-BENZODIAZEPIN-2-ONE	DERIVATIVE
H	H	Cl	CLONAZEPAM [A]	METHYL CLONAZEPAM [A']
H	OH	Cl	3-HYDROXY-CLONAZEPAM [B]	3-HYDROXY METHYL CLONAZEPAM
CH_3	H	F	FLUNITRAZEPAM [I]	— NO REACTION —
CH_3	OH	F	3-HYDROXY-FLUNITRAZEPAM [III]	— NO REACTION —
H	H	F	N-DESMETHYL-FLUNITRAZEPAM [II]	[I]
H	OH	F	3-HYDROXY-N-DESMETHYL-FLUNITRAZEPAM [IV]	[III]
H	H	H	NITRAZEPAM [G]	METHYL NITRAZEPAM [G']

Fig. 1. Chemical reactions of clonazepam, flunitrazepam, nitrazepam and some of their metabolites.

of specimens usually obtained from clinical studies. Therefore, a rapid yet sufficiently sensitive and specific assay was required to fulfil the requirements of assay automation.

The EC-GLC assay reported herein quantitates clonazepam and nitrazepam (G) (Table I) (used as a reference standard) as their methyl derivatives and is a modification of a published procedure for nitrazepam[23].

Flunitrazepam (I) and its N-desmethyl metabolite (II) are assayed as their intact moieties without derivatization using methylnitrazepam (G') (Table I) as the reference standard.

The sensitivity and specificity of the assay fulfil the requirements for its use in clinical pharmacokinetic evaluation. The EC-GLC assay can quantitate 0.5 to 1.0 ng of each compound per milliliter of blood and was used to measure blood levels of clonazepam in man following single oral 2-mg doses and following chronic oral

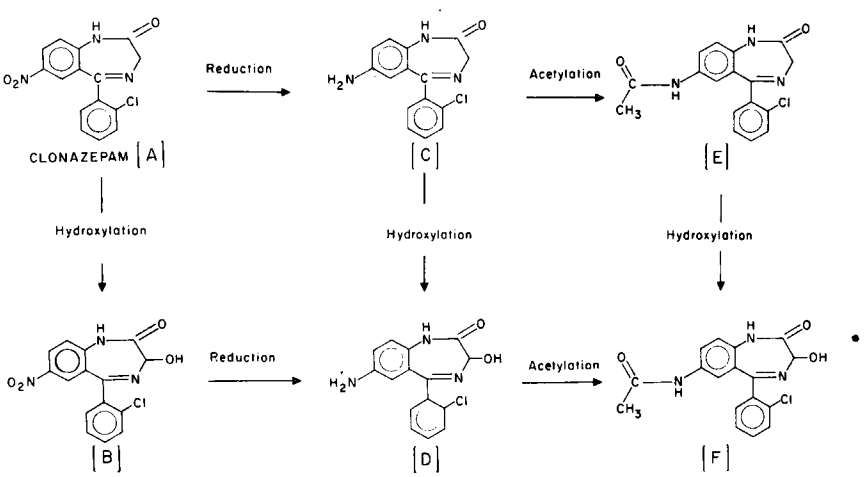


Fig. 2. Postulated major pathways of clonazepam biotransformation in man according to Eschenhof[12].

TABLE I

CHEMICAL NAMES AND PHYSICAL PROPERTIES OF CLONAZEPAM, FLUNITRAZEPAM, AND THEIR METABOLITES REFERRED TO IN FIG. 1

Compound	*Chemical name*	*Mol. wt.*	*M.p. (°C)*
Clonazepam series			
A	7-Nitro-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-(1H)-one (clonazepam)	315.70	238–240
A′	7-Nitro-5-(2-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-(1H)-one (methyl-clonazepam)	329.75	194–195
B	7-Nitro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-(1H)-one·hemiacetonate	361.0	159–160
C	7-Amino-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-(1H)-one	285.74	230–232
D	7-Amino-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-(1H)-one	301.74	340 (decomp.)
E	7-Acetamido-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-(1H)-one	322.77	292–300
F	7-Acetamido-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-(1H)-one	343.77	187–190 (decomp.)
G	7-Nitro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-(1H)-one (nitrazepam)	281.0	224–226
G′	7-Nitro-5-phenyl-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-(1H)-one (methylnitrazepam)	295.0	156–157
Flunitrazepam series			
I	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (flunitrazepam)	313.29	170–171.5
II	7-Nitro-5-(2-fluorophenyl)-3H-1,4-benzodiazepin-2-(1H)-one	299.77	210–211
III	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2H-1,4-benzodiazepin-2-one	329.30	216–217
IV	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one	315.28	193–195

administration of divided doses of 12 mg per day. Flunitrazepam blood levels following a single 2.0-mg oral dose in man were also measured.

EXPERIMENTAL

Conditions for EC-GLC analysis of clonazepam and flunitrazepam

Column. The column was a U-shaped 4 ft. × 4 mm I.D. borosilicate glass column, containing 3% OV-17 on 60–80 mesh Gas-Chrom Q (Applied Science Labs., State Park, Pa., U.S.A.). The column was conditioned as previously described[24].

Instrumental parameters. A MicroTek MT-220 gas chromatograph (Tracor Instruments, Austin, Texas, U.S.A.) equipped with a 15-mCi ^{63}Ni electron-capture detector was used.

The carrier gas used was argon–methane (90:10) (Matheson; oil pumped and dry), the column head pressure was set at 40 p.s.i.g., the flow-rate was 75 ml/min,

and the detector purge was 20 ml/min. The temperature settings were as follows: oven, 240°; injection port, 270°; detector, 320°. The conditions of column head pressure, flow-rate, and oven temperature must be adjusted so as to obtain a retention time (R_t) of 9.0 and 11.5 min for methylnitrazepam (G′) and methylclonazepam (A′), respectively (Fig. 3). Under these same conditions the retention times of flunitrazepam (I), its N-desmethyl metabolite (II), and methylnitrazepam (G′) (the reference standard) are 7.0, 11.5, and 9.0 min, respectively (Fig. 4).

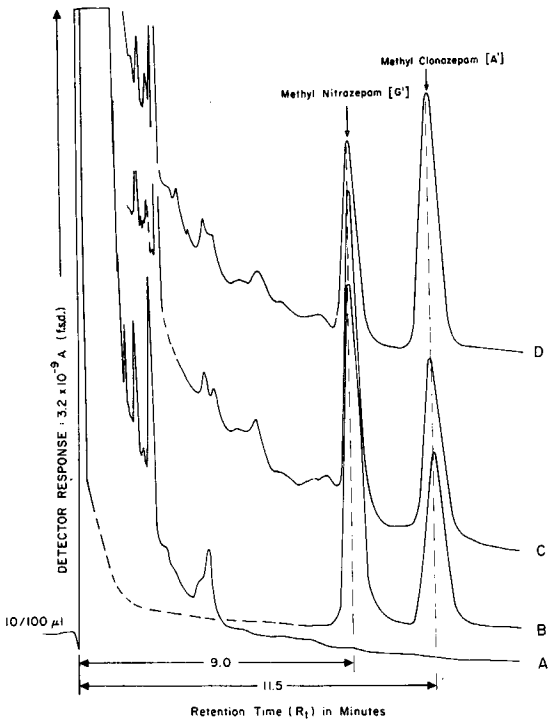


Fig. 3. Chromatograms of benzene–methylene chloride (90:10) extracts of: (A) control blood, (B) authentic methylclonazepam and methylnitrazepam standards, (C) control blood containing added clonazepam and nitrazepam following methylation, and (D) patient blood 2 h post oral dose of 2 mg of clonazepam.

The solid-state electrometer (Model No. 8169) input was set at 10^2 and output attenuation was 32 giving a response of 3.2×10^{-9} A full scale deflection (f.s.d.). The time constant on the 1.0-mV Honeywell recorder (Model No. 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 the methyl derivatives (A′) and (G′) showed maximum sensitivity at 46 V d.c. at a 270-μsec pulse rate and a 4-μsec pulse width. Under these conditions 1.0 ng each of methylnitrazepam (G′), methylclonazepam (A′), and flunitrazepam (I) and 2.5 ng of the N-desmethyl metabolite (II) give nearly full-scale pen response on the 1.0-mV recorder. The minimum detectable amount of A′, G′, I, and II is 0.5 to 1.0 ng of each per milliliter of blood.

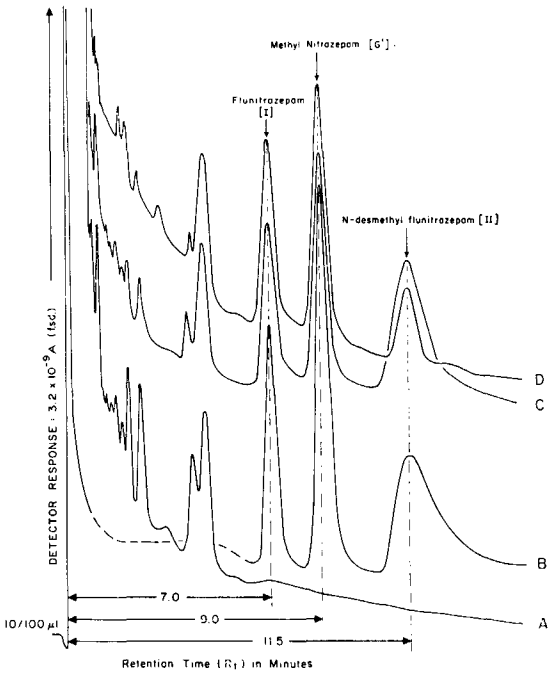


Fig. 4. Chromatograms of benzene–methylene chloride (90:10) extracts of: (A) control blood, (B) authentic flunitrazepam, methylnitrazepam and N-desmethyl metabolite standards, (C) internal standards of I, II, and methylnitrazepam (G') recovered from blood, and (D) patient blood 2 h post oral dose of 2 mg of flunitrazepam.

Preparation of standard solutions of the benzodiazepin-2-ones

The respective 7-nitro-1,4-benzodiazepin-2-ones clonazepam (A), nitrazepam (G), flunitrazepam (I), its N-desmethyl metabolite (II), methylclonazepam (A'), and methylnitrazepam (G'), which are required as analytical standards, are listed in Table I.

Weigh out 10.00 mg each of the above five compounds into separate 10-ml volumetric flasks. Dissolve, using 1.0 ml of absolute ethanol followed by 1 ml of acetone and then make up to volume with 20% acetone in benzene. These stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions by suitable dilutions in benzene–acetone–methanol (80:15:5), 100-µl aliquots of which are added to blood as internal standards.

For the clonazepam assay, standard solutions containing 2.0 to 10.0 ng of clonazepam (A) each containing 10.0 ng of nitrazepam (G) (as the reference standard) per 100 µl of benzene–acetone–methanol (80:15:5) are prepared.

For the flunitrazepam assay, standard solutions containing 2.0 to 10.0 ng each of flunitrazepam (I) and its N-desmethyl metabolite (II) each containing 10.0 ng of methylnitrazepam (G') (as the reference standard) per 100 µl of benzene–acetone–methanol (80:15:5) are similarly prepared.

External standard solutions for EC-GLC analysis

The following standard solutions are prepared from their respective stock solutions previously described. For the clonazepam assay, prepare working solutions

containing 2.0 to 10.0 ng of methylclonazepam (A') each containing 10.0 ng of methylnitrazepam (G') (the reference standard) per 100 μl of benzene–acetone–methanol (80:15:5). For the flunitrazepam assay, the standard solutions described above for flunitrazepam are used.

In each analysis 10-μl aliquots of the respective working solutions (equivalent to 0.2 to 1.0 ng of compound) are injected into the gas chromatograph to establish the detector response and linearity of the EC-GLC system for each day's analysis. The external standard (calibration) curves for both assays are prepared after the injection of two or three 10-μl aliquots of the control blood extract. In practice, 10-μl injections of the external standards are made between every two or three consecutive biological samples. Priming the column with the biological extract deactivates "reactive" sites on the column thereby reducing adsorption losses on the column especially of II, resulting in a significant improvement in its peak shape and electron-capture detector response.

Reagents

All reagents were of analytical grade (ACS) purity (>99%) and were used without further purification except where indicated. The inorganic reagents were made up in doubly distilled water and included 1.0 *M* H_3BO_3–Na_2CO_3–KCl buffer solution (pH 9.0) prepared as described[24], and 0.1 *N* NaOH. Tetrabutylammonium hydrogen sulfate (TBA) (Aldrich, Milwaukee, Wisc., U.S.A.; mol. wt. = 339.56, m.p. = 169–172°) has to be purified by double recrystallization from acetone to yield pure white crystals (m.p. 171–172°). Prepare a fresh 0.025 *M* solution of TBA in 0.1 *N* NaOH (8.5 mg/ml) prior to each assay.

The organic reagents used were: benzene and methylene chloride (nanograde; Mallinckrodt, St. Louis, Mo., U.S.A.), acetone and methanol (Fisher Scientific, Pittsburgh, Pa., U.S.A.), benzene–methylene chloride (90:10) as the solvent for extraction, benzene–acetone–methanol (80:15:5), stored over anhydrous sodium sulfate, and methyl iodide (Aldrich).

Extraction of clonazepam

Into a 15-ml centrifuge tube containing 10.0 ng of nitrazepam (reference standard) add 1.0 ml of blood and 2.0 ml pH 9.0 borate buffer, mix well and denature by intermittant heating in a boiling water-bath for no more than 30 sec, or until the sample just forms a rust-brown colored precipitate. Cool to room temperature, add 6 ml of benzene–methylene chloride (90:10), seal the tube with a PTFE stopper and extract by shaking for 5 min on a reciprocating shaker. Centrifuge the samples for 15 min at 2600 rpm (1500 *g*)* (preferably in a refrigerated centrifuge at 10°). Transfer a 5-ml aliquot into a fresh 15-ml centrifuge tube. Along with the samples process a specimen of control blood (taken from the subject prior to medication) and four 1-ml specimens of control blood 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 nitrazepam added as the reference standard. To the benzene–methylene chloride extract directly add 50 μl of CH_3I, and 0.50 ml of a 0.025 *M* solution of TBA in 0.1 *N* NaOH. Shake for 10 min at

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

room temperature on a reciprocating shaker*. Centrifuge the samples and remove the lower aqueous layer with a hypodermic syringe fitted with a cannula (Becton-Dickinson No. 20, 15 cm in length).

Wash the organic layer twice with 1 ml of distilled water to remove any contaminating reagent (TBA in 0.1 *N* NaOH). Remove the first water wash with the syringe, and transfer the organic phase after the second water wash into a fresh 15-ml centrifuge tube. Evaporate to dryness at 60° in the water-bath of a N-EVAP Model No. N-07 evaporator (Organomation Associates, Worcester Mass., U.S.A.) under a stream of pure dry nitrogen. Vacuum dry the residue (over Drierite) in a vacuum desiccator for 10 min to remove all traces of moisture, dissolve the residue in 100 μl of benzene–acetone–methanol (80:15:5) and inject a suitable aliquot (5 to 10 μl) for EC-GLC analysis.

Extraction of flunitrazepam and its N-desmethyl metabolite

Perform the extraction procedure as described above for clonazepam, using 10.0 ng of methylnitrazepam (G') added as the reference standard into each of the unknowns. Process these 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 with each tube containing 10.0 ng of methylnitrazepam added as the reference standard. The benzene–methylene chloride (90:10) extract is evaporated to dryness at 60° under a stream of pure nitrogen, the residue is vacuum dried, dissolved in 100 μl of benzene–acetone–methanol (80:15:5), and a suitable aliquot (10 μl) is analyzed by EC-GLC as described.

The peaks due to the respective benzodiazepin-2-ones are identified by their respective retention times (Figs. 3 and 4), and their peak areas determined by measuring peak height (cm) $\times$ width at half height (cm) using the slope baseline technique, or by electronic digital integration.

Quantitation of clonazepam (A), flunitrazepam (I), and its N-desmethyl metabolite (II) by EC-GLC analysis

Calibration curves using either the relative standard method (peak area ratio *vs.* concentration) as shown for clonazepam in Fig. 5A or the direct calibration method (peak area *vs.* concentration) as shown for flunitrazepam (I) in Fig. 5B, can be used for the quantitation of either compound in the unknowns.

If the relative calibration (peak area ratio) method is used (Fig. 5A), the concentration in the unknowns is read directly (by interpolation) from the calibration curve. However, if the direct standard (peak area) method is used (Fig. 5B), then correction factors for the aliquot injected, the overall recovery of added internal standards, and any dilutions incurred, have to be made.

The concentration of each component per milliliter of sample analyzed is calculated as previously described[21].

Determination of per cent recovery

The overall recovery of clonazepam (A), flunitrazepam (I) and its N-desmethyl metabolite (II) are determined by using the direct calibration (peak area *vs.* concen-

* Eberbach, Ann Arbor, Mich., U.S.A.; at about 80–100 strokes per minute.

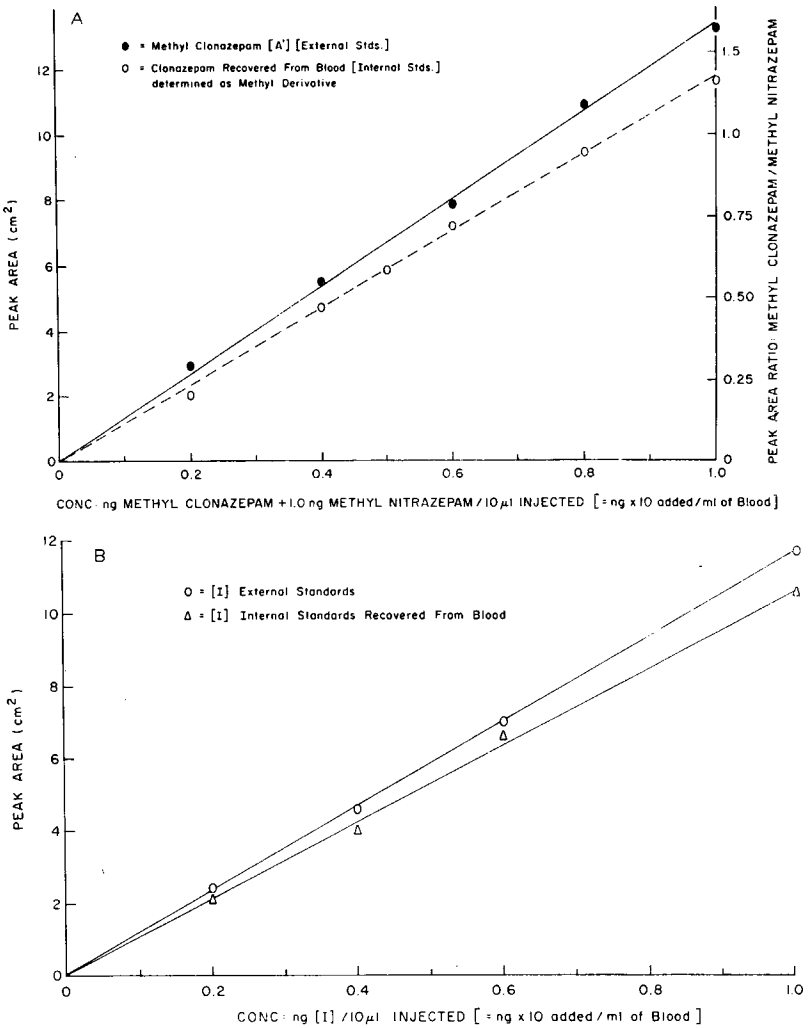


Fig. 5. Electron-capture detector calibration curves using (A) the relative standard method (peak area ratio *vs.* concentration) shown for clonazepam, and (B) the direct standard method (peak area *vs.* concentration) as shown for flunitrazepam.

tration) method as shown for flunitrazepam (Fig. 5B). When using the relative calibration (peak area ratio) method (Fig. 5A), the recovery of the respective compounds measured remains constant because irrespective of the actual recovery the ratio of the two peaks is constant thereby compensating for any physical losses incurred due to sample processing.

RESULTS AND DISCUSSION

Sensitive and specific EC-GLC assays have been reported for the determination of medazepam[24], chlordiazepoxide[25], diazepam[24,26,27], nitrazepam[23], and temazepam[28], as the intact compounds, and for diazepam[29,30], bromazepam[31], oxazepam[32,33],

lorazepam[33], clonazepam, flunitrazepam[21], nitrazepam[34], and temazepam[35], as their respective *o*-aminobenzophenone derivatives produced by acid hydrolysis. In all these methods the compound of interest was extracted into a suitable solvent from blood or plasma buffered above pH 7.0, back extracted into acid (in which it was also hydrolyzed where needed), re-extracted into diethyl ether following alkalization of the medium, and the ether residue was analyzed by EC-GLC.

In the case of clonazepam, flunitrazepam[21], and nitrazepam[34], it was necessary to convert them to the benzophenone by acid hydrolysis because the parent compound was either unstable in acid and/or required high temperatures (>250°) for elution from a suitable column, usually also with prolonged "tailing" of the peak. The benzophenone derivatives can be analyzed with subnanogram sensitivity as well-resolved Gaussian-shaped peaks at column temperatures within the bleed limits of the liquid phase employed[21]. The above procedures are all time consuming and not readily amenable for the rapid, automated analysis of the numbers of specimens obtained from large-scale clinical studies. It was apparent that different extraction procedures resulting in minimum sample contamination and/or clean-up required, and suitable derivatization methods for quantitation by EC-GLC at subnanogram concentrations were highly desirable. The N-1-desalkylbenzodiazepin-2-ones can be derivatized by methylation using CH_3I as reported for nitrazepam[23], or silylated to form trimethylsilyl (TMS) derivatives[27]. These derivatives can be chromatographed to yield symmetrical peaks with good sensitivity to the electron-capture detector. The N-1-methyl derivatives are preferred to the N-1-TMS derivatives since they are more temperature stable and are not hydrolyzed by traces of moisture as are the latter. The analysis of the 1,4-benzodiazepin-2-ones as the intact moiety is also preferred to analysis by hydrolysis as their *o*-aminobenzophenones because of greater specificity obtained. Consequently, assays that utilized the benzophenones as the derivative for analysis[29–35] are being modified utilizing derivatization techniques which render the intact benzodiazepines more amenable to GLC analysis.

The simplified extraction procedure described is limited to 1 ml or less of blood per analysis and has a limit of detection of 0.5 to 1.0 ng/ml. It was used to measure blood levels following single oral doses and chronic oral dosing where blood levels have reached steady state. The higher drug levels in the latter situation permit the use of small sample volumes (100 μl), and usually require further dilution of the final residue, when impurities and other minor metabolites are diluted out leaving either the parent drug and/or its major metabolites for quantitation.

The analysis of intact clonazepam using nitrazepam as the reference standard requires derivatization of the compounds using CH_3I to form methylclonazepam (A′) and methylnitrazepam (G′), respectively. The two compounds are eluted as completely resolved Gaussian-shaped peaks (Fig. 3) at relatively low column temperatures (240°) and can be quantitated with picogram sensitivity.

The present method is a modification of a published procedure for nitrazepam[23], wherein the reaction conditions have been refined with respect to time of reaction and concentration of the reactants. The commercial grade of TBA has to be recrystallized twice to yield pure white prismatic crystals (m.p. 171–172°) before use. Studies on the kinetics of the methylation reaction indicated that optimal and reproducible methylation was obtained at 25° using CH_3I catalysed with a 0.025 *M* solution of TBA in 0.1 *N* NaOH. The methylation of clonazepam proceeds to

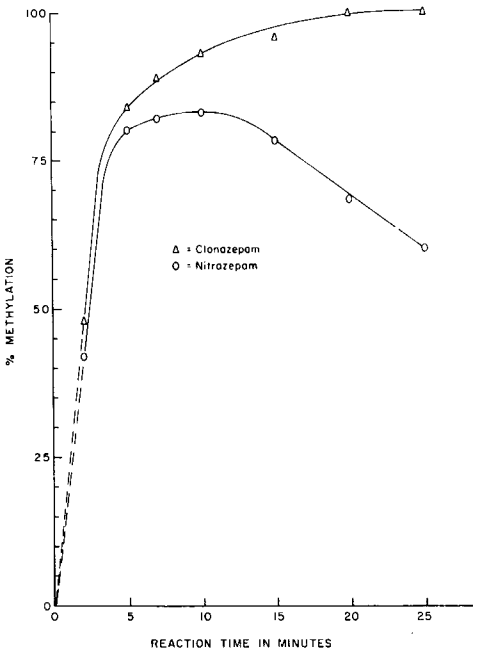


Fig. 6. Time course curve of the methylation of clonazepam and nitrazepam.

completion very rapidly (Fig. 6) and yields >90% of the methyl derivative in 10 min. The methylation of nitrazepam appears to peak at 10 min (85% yield) but declines thereafter. This may be due either to the preferential methylation of clonazepam, or an insufficient concentration of the reactants to drive the reaction for nitrazepam to completion. Although methylene chloride was reported to be a better solvent than benzene for methylation[23], it is apparent that the mixture of benzene–methylene chloride (90:10) is equally effective. The residues of biological specimens processed by this method yield chromatograms devoid of extraneous impurities in the retention areas of interest (Fig. 3) resulting in baseline resolution of the peaks for accurate quantitation. These modifications have resulted in more reproducible and quantitative methylation of clonazepam and nitrazepam in nanogram concentrations in biological extracts which yield chromatograms devoid of major impurities which would otherwise seriously contaminate the ^{63}Ni electron-capture detector and thereby impair quantitation.

Recovery

The overall recovery of varying amounts of clonazepam (2.0 to 50 ng) added per milliliter of blood determined by EC-GLC (after methylation) and by scintillation radiometry using ^{14}C-labeled clonazepam was of the order of 90 ± 5.0%. The recoveries of nitrazepam (G) (after methylation) and of methylnitrazepam (G') from blood determined by EC-GLC were 85 ± 5.0% and 95 ± 5.0%, respectively. The overall recovery of flunitrazepam (I) and its N-desmethyl metabolite (II) added in varying amounts (2 to 10 ng/ml) to blood and determined by EC-GLC analysis was 90 ± 5.0% for I and 80 ± 5.0% for II. The peak due to the N-desmethyl metabolite II when chromatographed as an authentic standard in the absence of a blood extract

shows considerable "tailing". This "tailing" effect is diminished in the presence of a blood extract and resulted in an enchanced Gaussian-shaped peak which enables more precise peak area integration (Fig. 4). This behaviour is probably due to the formation of an adsorption complex of the extracted lipids with active sites on the column thereby reducing adsorption losses during EC-GLC analysis resulting in enhanced recovery and a symmetrical peak shape for II (Fig. 4). The use of lecithin (a phospholipid) as a priming agent for deactivating adsorption sites on a column has been reported for the electron-capture determination of steroids at the picogram level[36].

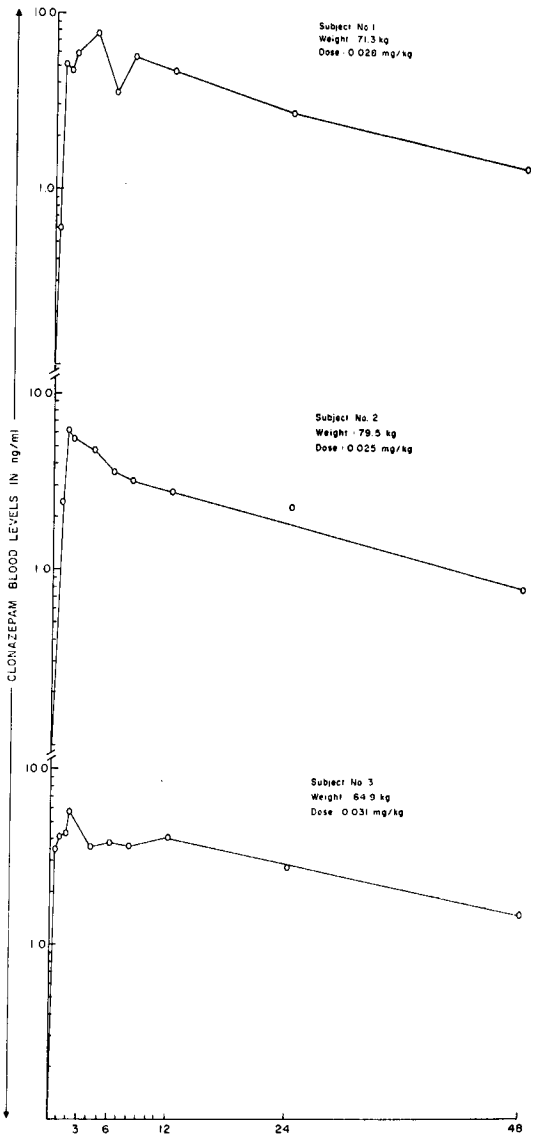


Fig. 7. Blood level fall-off curves of intact clonazepam (ng/ml) following a 2-mg oral dose of the drug in a tablet formulation.

Specificity of the assay

Both clonazepam and flunitrazepam undergo metabolic pathways which are analogous to nitrazepam[37,38] (Fig. 2). Only the parent compounds and metabolites containing the 7-nitro group (Fig. 1) would be expected to give good EC-GLC response. The 7-amino and 7-acetamido metabolites of nitrazepam were reported to yield very poor electron-capture detector response[23]. This finding also pertains to the corresponding metabolites of clonazepam and flunitrazepam notwithstanding the presence of the 2'-halogen (Cl or F) in these compounds. Hence these compounds are not measured by the present assay. Consequently, only the 7-nitro compounds are quantitated by EC-GLC analysis. The 7-nitro-3-hydroxy metabolites of clonazepam, and of flunitrazepam (B, III and IV) are resolved from their respective parent drugs by shorter retention times, hence, they do not interfere with the quantitation of either clonazepam or flunitrazepam. These metabolites are, however, non-measurable in blood and are also minor urinary constituents[12]. The anomalous phenomenon of a shorter retention time on a polar column, OV-17, of the more polar 3-hydroxy metabolites, especially metabolites B and IV, suggests thermolytic rearrangement under the EC-GLC conditions used. The thermolytic rearrangement of 3-hydroxy-N-desalkyl-1,4-benzodiazepin-2-ones such as oxazepam and lorazepam to yield their respective quinazoline-carboxaldehydes was demonstrated by gas chromatographic–mass spectrometric analysis[39,40].

Application of the method to biological specimens

Clonazepam. Blood levels of clonazepam were measured in three human subjects following a single oral 2-mg dose of the drug from the clinical tablet formulation. Blood levels were measurable up to 48 h post dosing and ranged from a maximum of 6.0 to 9.0 ng/ml at 1.5 to 2 h, to about 1.0 ng/ml at 48 h (Fig. 7). Blood levels were also monitored in a single patient undergoing chronic clonazepam therapy in divided daily doses totalling 12 mg per day for the management of petit mal seizures. The blood levels ranged from 39.6 to 77.1 ng/ml (Table II) over a one-month period, and appeared to show steady-state levels during this period.

Flunitrazepam. Blood levels of flunitrazepam were measured in one subject following a single 2-mg oral dose of the drug in a tablet. Blood levels were measurable

TABLE II

PLASMA LEVELS OF CLONAZEPAM IN MAN FOLLOWING CHRONIC ADMINISTRATION

Subject: M.B. (f); dose: variable —divided daily doses of 12 mg; weight: 22 kg.

Date of sampling	*Time drawn*	*Total daily dose (mg)*	*Plasma concentration (ng/ml)*
10/24/73	8:00 a.m.	12	40.0
10/25/73	8:00 a.m.	12	39.6
10/26/73	12:30 p.m.	12	53.5
10/27/73	12:30 p.m.	12	54.0
10/28/73	10:00 a.m.	12	56.3
10/30/73	11:30 a.m.	12	77.1
11/5/73	11:50 a.m.	12	64.9
11/14/73	10:15 a.m.	12	46.0

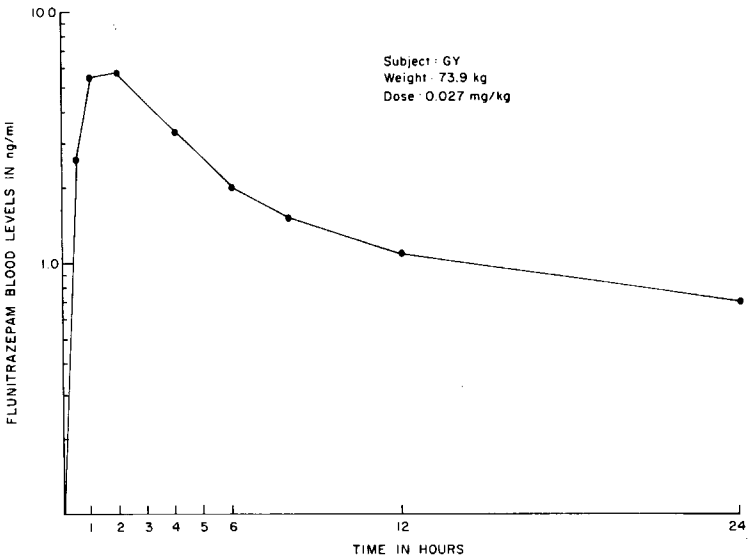


Fig. 8. Blood level fall-off curve of flunitrazepam (ng/ml) following a 2-mg oral dose of the drug in a tablet formulation.

from 30 min to 24 h post dosing reaching a maximum of 5 to 6 ng/ml between 1 to 2 h (Fig. 8). The blood levels at 48, 72, and 96 h were below the limit of measurement of both the direct assay and by hydrolysis to the benzophenone[21]. Trace amounts of the N-desmethyl metabolite (II) were seen only between 2 to 4 h post dosing. It is expected that steady-state blood levels of flunitrazepam and its N-desmethyl metabolite (II) can be more readily measured following chronic administration.

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