Environmental Health Perspectives
Vol. 36, pp. 69-76, 1980

Methane Negative Chemical Ionization Analysis of 1,3-Dihydro-5-phenyl-1,4-benzodiazepin-2-ones

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The methane negative chemical ionization (NCI) mass spectra of the medically important 1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-ones generally consisted solely of M^- and $(M-H)^-$ ions. Attempts to find the location of the H^- lost in the generation of the $(M-H)^-$ ion were unsuccessful, although many possibilities were eliminated. A Hammett correlation analysis of the relative sensitivities of a series of 7-substituted benzodiazepines suggested that the initial ionization takes place at the 4,5-imine bond. For certain benzodiazepines, the $(M-H)^-$ ion generated by methane NCI was 20 times more intense than the MH^+ ion generated by methane positive chemical ionization (PCI). By using NCI, a sensitive and simple GC-MS assay for nordiazepam was developed that can quantitate this important metabolite of many of the clinically used benzodiazepines in the blood and brain of rats.

Introduction

Compounds with the general 1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-one structure (Table 1) are extensively used in medicine as anxiolytic (Valium, Librium, Serax, Ativan, Verstran, Tranxene), anticonvulsant (Clonopin) and hypnotic (Dalmane, Mogadon, Rohypnol) agents (*1*). Because of the high potency of these compounds and their large volume of distribution (*2*), they are present, following dosing, at relatively low concentrations in blood.

Fortunately for analytical purposes, most of these compounds have suitable characteristics for analysis by gas chromatography (GC) and excellent electron capture (EC) properties. Thus, most assays for these compounds have used EC-GC to obtain the required sensitivity (*3-5*). However, in certain circumstances, eg., with the small volume of blood available from children or infants with epilepsy or with blood from single-dose experiments, the sensitivity using EC-GC is often insufficient. In addition, the EC-GC assays are often difficult to use in the analysis of these chemicals at their site of action, brain tissue, because of interference from coeluting substances.

For these reasons, i.e., the need for greater sensitivity and specificity, we investigated the applicability of the NCI technique, which has been pioneered by Dougherty and Hunt (*6-9*), to the analysis of the 1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-ones. From a consideration of their EC response, this class of compounds should be ideally suited to analysis by EC-NCI. Recently, we modified our PCI GC-MS assay for Clonopin to measure this compound by EC-NCI (*10*). For Clonopin, we found NCI to be approximately twenty times more sensitive than PCI with less complex ion chromatograms. To date, NCI assays have been published for melatonin (*11*) and 2,3 dibromopropanol (*12*).

In this paper, we report the NCI mass spectra of a number of substituted 1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-ones, a study of their EC-NCI sensitivity as a function of structure and a GC-NCI-MS assay for nordiazepam, a common metabolite of many clinically used benzodiazepines. The assay's limit of sensitivity is 1 ng or less of nordiazepam in either 1 g of rat brain or 1 ml of rat blood.

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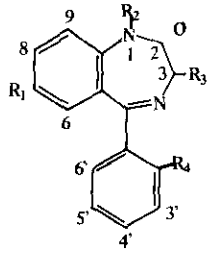
Table 1. Structures of a few clinically used 1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-ones and their numbering system.



R_1	R_2	R_3	R_4	Name	Trade name
Cl	CH_3	H	H	diazepam	Valium
Cl	H	H	H	nordiazepam	———
NO_2	H	H	Cl	clonazepam	Clonopin
Cl	$CH_2CH_2N(CH_2CH_3)_2$	H	F	flurazepam	Dalmane
NO_2	H	H	H	nitrazepam	Mogadon
NO_2	CH_3	H	F	flunitrazepam	Rohypnol
Cl	CH_2-CH(-CH_2-CH_2)	H	H	prazepam	Verstran
Cl	H	OH	H	oxazepam	Serax
Cl	H	OH	Cl	lorazepam	Ativan
Cl	H	CO_2K (KOH)	H	clorazepate	Tranxene
Cl	CH_3	OH	H	temazepam	Levanxol

Material and Methods

Instrumentation

Selected ion monitoring measurements were made using a Finnigan model 3200 quadrupole mass spectrometer equipped with a Finnigan Promim peak monitor. Mass spectra were obtained using a Finnigan model 1015 quadrupole mass spectrometer and Finnigan model 6000 data system. Both mass spectrometers were coupled to a Finnigan model 9500 gas chromatograph and were modified to detect negative ions using the method of Stafford et al, (*13*). The conversion dynode was a dynode from a Finnigan discrete stage electron multiplier. The voltage to the conversion dynode was typically +2500 V. The electron multiplier, a Galileo model 4770, was typically operated at −1800 V. Separate power supplies were used to provide the ion energy and lens voltages. The trap and ion repeller were electrically tied to the ion energy. A 600 gauss magnet was placed outside the vacuum on the quadrupole housing either above the ion source (Finnigan 3200) or above the electron multiplier (Finnigan 1015). The magnet was moved until the small methane NCI background signal at *m/e* 35 (^{35}Cl) was at a maximum. The ion energy and lens voltages and the quadrupole rod polarity were set to give the most intense signal consistent with useful peak shape and unit mass resolution. The filament emission and electron energy were set to their lowest values consistent with maximum negative ion production. The ion source temperature was between 90 and 130°C.

Each Promim channel was operated with a 100 msec dwell time, a 0.5 Hz frequency response, and a gain of 10^8 V/A. Ion chromatograms were recorded on a four pen Rikadenki recorder using a paper speed of 2 cm/min. To record mass spectra, the model 6000 data system set the mass spectrometer to scan every 2 sec between *m/e* 10 and *m/e* 410 with a 5 msec/amu integration time. The signal threshold was set at 1 bit.

The GC column, 4 ft × 2 mm inside diameter, was packed with 3% OV-17 on 120-140 mesh Gas Chrom Q (Applied Science Laboratories). Prior to use, the column was conditioned at 300°C overnight with a 20 ml/min nitrogen flow. For the assay the temperature of the injector, the column oven, the separator oven, and the source re-entrant tube were 300, 280, 250, and 250°C, respectively. Methane was used both as GC carrier gas (10 psi) and CI reagent gas. Under these conditions the retention time of nordiazepam was 120 sec.

For both GC-MS and direct insertion probe (DIP)-MS determinations, the methane ion

source pressure was 0.3–0.5 torr. This value was determined by MacLeod gauge connected to the ion source by a modified DIP.

Materials

Heptane, acetone, methanol, and dichloromethane were all of nanograde quality (Burdick and Jackson). 1,2-Dichloroethane was analytical grade (Fisher Scientific). The preparation of 1*M* pH 10 borate buffer has been described (*14*). Water was distilled. Methane (ultra high purity) was obtained from Matheson Gas Products. Culture tubes with Teflon-lined screw caps (16 ml, Pyrex 9825) were used for the blood or brain homogenate extractions. Centrifuge tubes (5 ml, Pyrex 8061) were used for evaporation of the extracting solvent. Prior to use, all glassware was cleaned with detergent, treated with a 1% aqueous solution of Prosil-28 (PCR Research Chemicals) and consecutively rinsed with water, dichloromethane and methanol.

Unlabeled and deuterated benzodiazepines were obtained from Drs. W. Scott and A. Liebman, respectively, of the Chemical Research Department, Hoffmann-La Roche Inc., Nutley, N. J., 07110. Stock solutions (mg/ml) of all compounds were prepared in methanol. For the assay, several methanolic working solutions were prepared from the mg/ml stock solution of nordiazepam and [2′,6′-^{2}H]-nordiazepam (nordiazepam-d_2). Solutions A-F contained 0, 100, 250, 500, 1000 and 2000 ng/ml of nordiazepam, respectively, and 2000 ng/ml of nordiazepam-d_2.

Sprague Dawley male rats (250 g) were purchased from Charles River Inc. and were caged and fed for one week prior to use. The dosing solution (10 mg/ml) was prepared by dissolving nordiazepam in polypropyleneglycol. Three rats were given a 10 mg/kg oral injection of this solution. At the designated times the rats were anesthetized with ethyl ether, and blood was removed by open chest cardiac puncture. The blood was placed in a culture tube containing a small amount of heparin and frozen at −15°C. The rats were then decapitated and their brains removed. The brains were wrapped in aluminum foil, frozen on Dry Ice, and stored at −15°C.

Nordiazepam Assay Procedure

The frozen brains were weighed and homogenized (Tekmar tissumizer, 20 sec, 90 rpm) in 5 ml of water/ethanol (1:1). The cold blood sample tubes were placed in hot water until the blood thawed. Either 1 ml of blood or 1 ml of the brain homogenate was transferred to a 16 ml culture tube. To the tube was added 200 ng of nordiazepam-d_2 (0.1 ml of solution A) and 2 ml of 1*M* pH 10 borate buffer. The mixture was vortexed on a Vortex Genie, 6 ml of a solution of 20% 1,2-dichloroethane in benzene was added, and the sample was extracted by gently shaking for 30 min on a Eberbach variable speed reciprocating shaker. The tube was centrifuged (Damon model CRU-500) at 10°C for 10 min at 1500*g*. The organic phase was transferred by using a Pasteur pipet to a 5 ml centrifuge tube and the solvent removed at 60°C with a gentle stream of nitrogen (N-Evap, Organomation Assoc.). The residue was reconstituted in 100 μl of a solution of 10% acetone in heptane; 1–3 μl of this solution was then injected into the GC-MS. Approximately 45 sec after sample injection, the GC divert valve was closed to allow the GC effluent to enter the ion source. The ion source supplies were turned on 5 sec later.

Prior to injecting any samples, the GC-column was treated with several injections of the reconstituted residue from drug free rat brain extracts. Several 1 μl injections of solution F were made to tune the mass spectrometer by using the Promim, to monitor *m/e* 269, the $(M-H)^-$ ion of nordiazepam, and *m/e* 273, the ^{37}Cl isotope variant of the $(M-H)^-$ ion of nordiazepam-d_2. Additional injections of the same solution were made to find the optimum ion source voltages and the precise column oven temperature which gives nordiazepam a retention time of 120 sec. Aliquots of solutions A-F were then injected. A 0.1 μl injection of solution F should give a response at *m/e* 269 with a signal-to-noise ratio greater than 100. If this is not true, either the column needs to be replaced or the ion source and/or quadrupole rods need cleaning. Injection of 1 μl of solution A should give a response at *m/e* 269 which is less than 2% of the response at *m/e* 273. Injection of 5 μl of the 10% acetone in heptane solvent mixture should give no significant response at *m/e* 269 or 273.

The peak heights in the ion chromatogram were measured with a ruler and the *m/e* 269 to *m/e* 273 ion ratio calculated. The ratio was converted to a compound amount using a standard curve. The standard curve was generated from the analyses of the ion ratio versus nordiazepam added data from 1 ml specimens of either drug-free brain homogenate or drug-free blood each spiked with 100 μl of either solution A (0 ng), B (10 ng), C (25 ng), D (50 ng), E (100 ng), or F (200 ng). The data from the standards were fitted by a nonlinear least squares procedure to the standard isotope dilution equation (*15, 16*). The constants from the least-squares fitting were used to calcu-

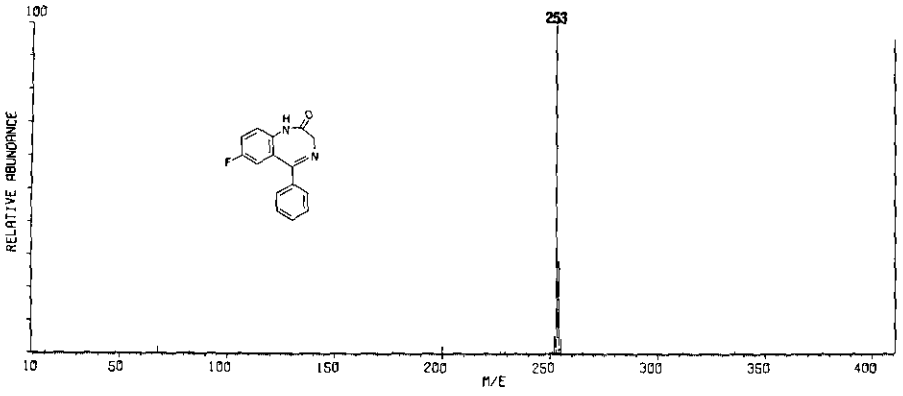


FIGURE 1. Methane NCI mass spectrum of 7-fluoro-1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-one (MW = 254).

late the concentration of nordiazepam in an unknown sample given a measured ion ratio.

Results and Discussion

The NCI mass spectrum of 7-fluoro-1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-one, shown in Figure 1, is representative of that obtained from this class of compound. The spectrum is quite simple, consisting principally of an intense $(M-H)^-$ ion and a much smaller M^- ion with virtually no other, even low molecular weight ions. Strong $(M-H)^+$ ions are observed in the positive electron ionization (PEI) mass spectra of this class of compounds (*17*), while MH^+ ions dominate their "acid-base" PCI mass spectra.

The M^- ion in the NCI spectra tended to be small except when R_1 is NO_2 (Fig. 2). In this case, the M^- ion tended to be as intense, or somewhat more intense, than the $(M-H)^-$ ion. At certain times over the last year, the M^- ion for several compounds NO_2-substituted at the R_1 position was 100 times more intense than their $(M-H)^-$ ion. At these same times, other 1,3-dihydro-5-phenyl-1,4 benzodiazepin-2-ones also showed enhanced M^- ion. The reason(s) for this is unclear and the problem is under investigation.

Since several specifically deuterated nordiazepam analogs were available, we tried to locate the origin of the $H^\cdot$ lost in the generation of the $(M-H)^-$ ion. We found that for nordiazepam specifically deuterated at the 6 position or dideuterated at the 2',6' positions no significant (e.g., 5%) loss of deuterium was observed. These results are quite different than those of Benz and Vane, who analyzed these same compounds by PEI (*18*). These workers found that 55–60% of the H lost to generate the $(M-H)^+$ ion in the PEI mass spectrum came from these positions.

The NCI mass spectra of nordiazepam specifically deuterated at position 9 or dideuterated at position 3 were obtained. Again no significant loss of deuterium was observed.

If the H lost was at position 1, methylation at the nitrogen should dramatically decrease the ratio of the $(M-H)^-$ to M^- ions. Instead, we found

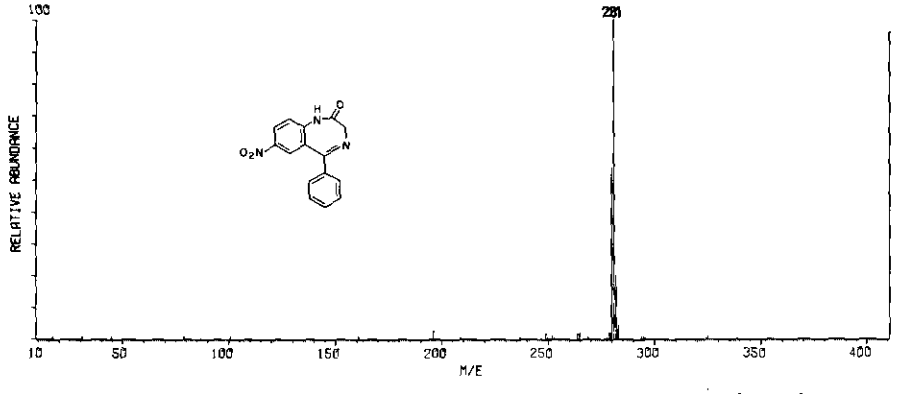
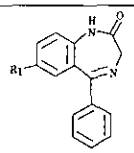


FIGURE 2. Methane NCI mass spectrum of 7-nitro-1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-one (MW = 281).

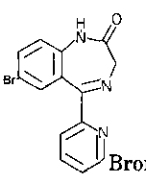
Table 2. Relative sensitivities of various 7-substituted 1,3-dihydro-5-phenyl-1,4 benzodiazepin-2-ones.



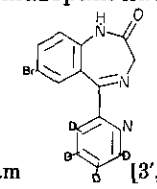
R_1	Direct insertion probe-mS	Gc-Ms
Cl	44.3	67.3
$COCH_3$	34.8	31.8
F	30.0	30.9
OCH_3	1.5	0.9
H	1.0	1.0
OH	0.9	—
CH_3	0.8	0.5
NH_2	0.1	0.2

that diazepam gave approximately the same ratio of $M\text{-}H^-$ to M^- ions as nordiazepam. To test the possibility that the influence of the N-methyl was being masked by the loss of one of the methyl hydrogens, we also obtained the NCI mass spectrum of [1-C^2H_3]-nordiazepam. However, this compound's mass spectrum also showed no loss of deuterium and thus we concluded that the hydrogen lost was not that at position 1.

No isotope variants of nordiazepam were available with deuterium at either 3′,4′, or 5′. However, [3′,4′,5′,6′-^{2}H]-bromazepam had been synthesized.



Bromazepam



[3′,4′,5′6′-^{2}H]-Bromazepam

The NCI spectrum of unlabeled bromazepam was dominated by generation of a Br^- ion. Fortunately, the spectrum also contained a $(M\text{-}H)^-$ ion with an intensity approximately 5% of the intensity of the Br^- ion and virtually no M^+ ion. Consistent with the noninvolvement of the hydrogens at the 3′, 4′ or 5′ positions in $(M\text{-}H)^-$ ion formation, the spectrum of [3′,4′,5′,6′-^{2}H]-bromazepam showed the loss of H^-, but no loss of deuterium.

By elimination, the 8-position is left as the location of the H^- lost. We are currently synthesizing nordiazepam deuterated at the 8-position to check

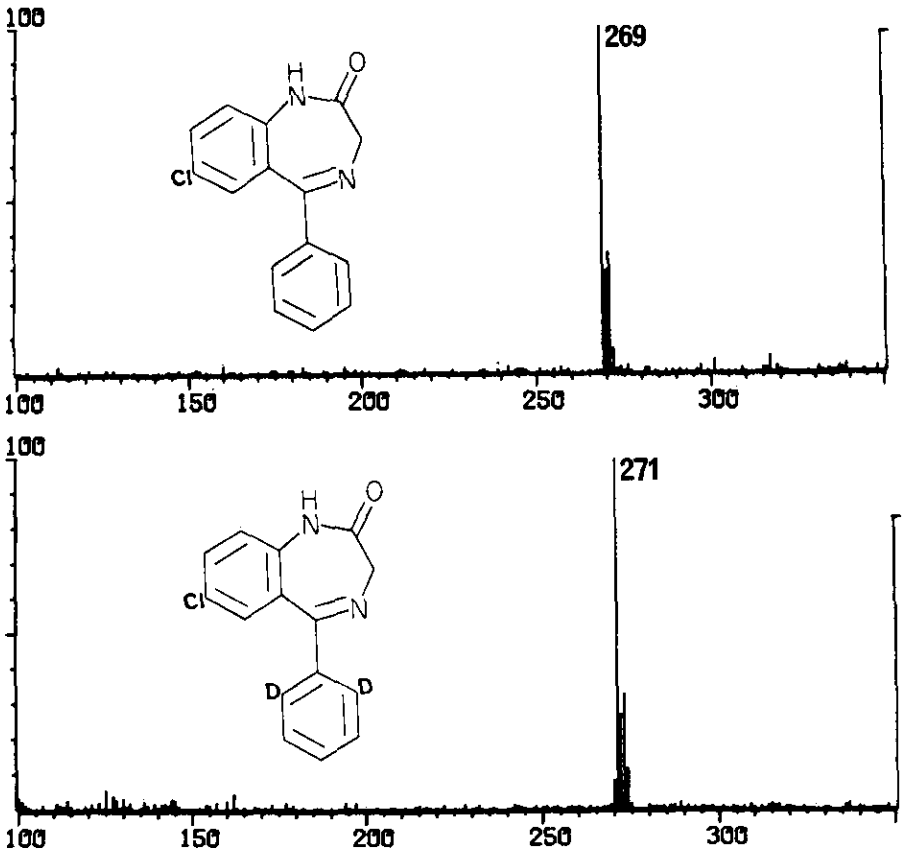


FIGURE 3. Methane NCI mass spectra of nordiazepam (upper spectrum, MW = 270) and nordiazepam-d_2 (lower spectrum, MW = 272).

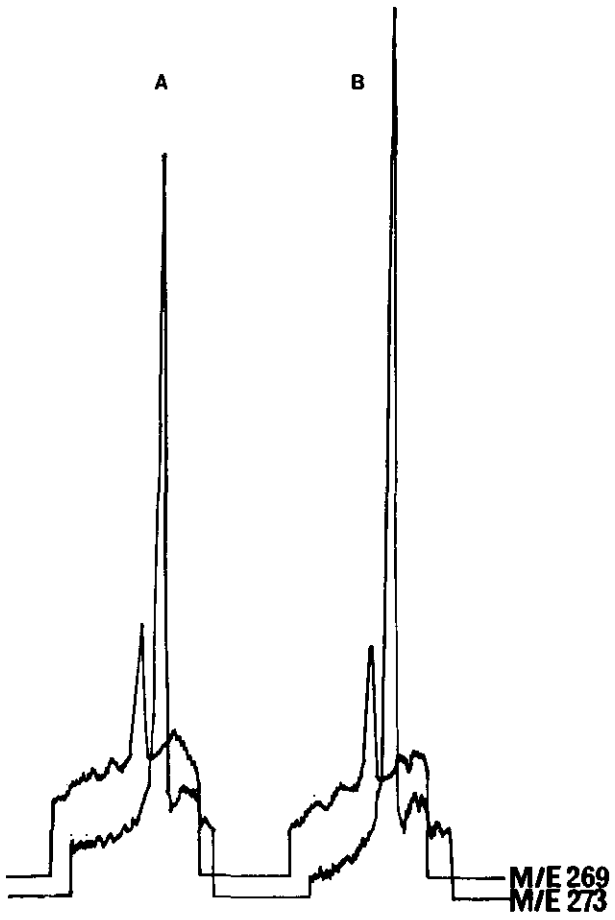


FIGURE 4. Ion chromatograms from the analysis of (A) 1 ml of rat brain homogenate and (B) 1 ml of rat blood, each spiked with 1 ng of nordiazepam and 10 ng of nordiazepam-d_2.

this possibility. In addition, we are synthesizing nordiazepam specifically deuterated at the 3′, 4′, and 5′ positions to verify the conclusion inferred from the deuterated bromazepam mass spectrum. If these compounds fail to establish the origin of the H lost, we may have to radically change our view of the nature of the $(M-H)^-$ ion.

It became apparent, after obtaining spectra on several benzodiazepines, that the NCI sensitivity of the various compounds varied greatly. In particular, the sensitivity varied as a function of the nature of the substituent at R_1. For instance nordiazepam was approximately 400 times more sensitive than 7-amino-1,3-dihydro-phenyl-1,4-benzodiazepin-2-one, when both compounds were analyzed by DIP-NCIMS. By methane PCI analysis, on the other hand, the sensitivities of both the compounds were approximately equal.

The NCI sensitivities of various 7-substituted 1,3-dihydro-5-phenyl-1,4-benzo-diazepin-2-ones relative to the 7-H compound are given in Table 2. Each data point was the average of two measurements of the intensity of all ions in the NCI mass spectra. Duplicate measurements were within 20% of one another. We could not chromatograph the compound with R_1 equals OH and this compound was not included as a data point in the GC-MS sensitivity comparison. The GC-MS analyses were performed by using the column and GC conditions described in the experimental section for the nordiazepam assay. The compound with a 7-NO_2 substituent gave erratic sensitivity and was not included. We attributed the variation to the tendency of this type of compound to undergo reduction in the CI ion source (*19*).

The logarithm of the relative sensitivities in Table 2 were highly correlated with the Hammett σ_{meta} values of the R_1 substituent (*20*). The equation, $y = (m \pm SD)\ (x) + (b \pm SD)$, for the σ_{meta} correlation was log (relative sensitivity) = (10.3 ± 0.74) $(\sigma_{meta}) + (-0.2 \pm 0.18)$. The correlation coefficient for the fit was 0.97. The correlation coefficient for the fit with the Hammett σ_{para} values of the R_1 substituent was only 0.82. The functional group *meta* to the 7-position is the 4,5 imine bond and our data suggests that this bond is the site of electron capture. This bond is also the suspected site of ionization in the polarographic analysis of 1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-ones (*21*). The suggestion that the imine bond is the site of ionization is also supported by the effect of fluorine substitution in the 5-phenyl ring on NCI sensitivity. Compared to the sensitivity of nordiazepam, 3′-fluoronordiazepam was found to be significantly more sensitive than 4′-fluoronordiazepam. This is consistent with ionization at the

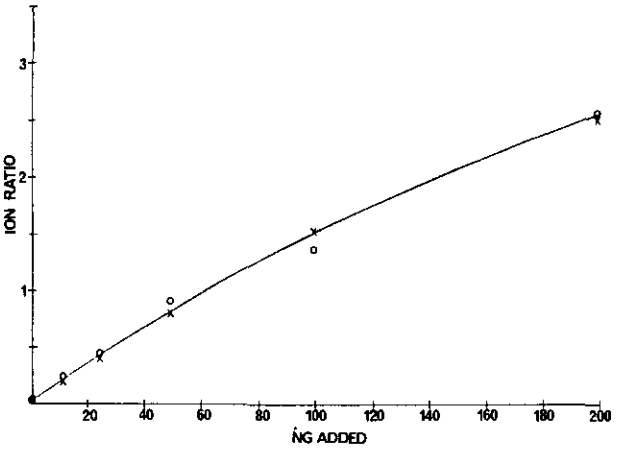


FIGURE 5. Typical standard curve from the analysis of (×) blood and (○) brain homogenate, each spiked with various amounts of nordiazepam and 200 ng of nordiazepam-d_2.

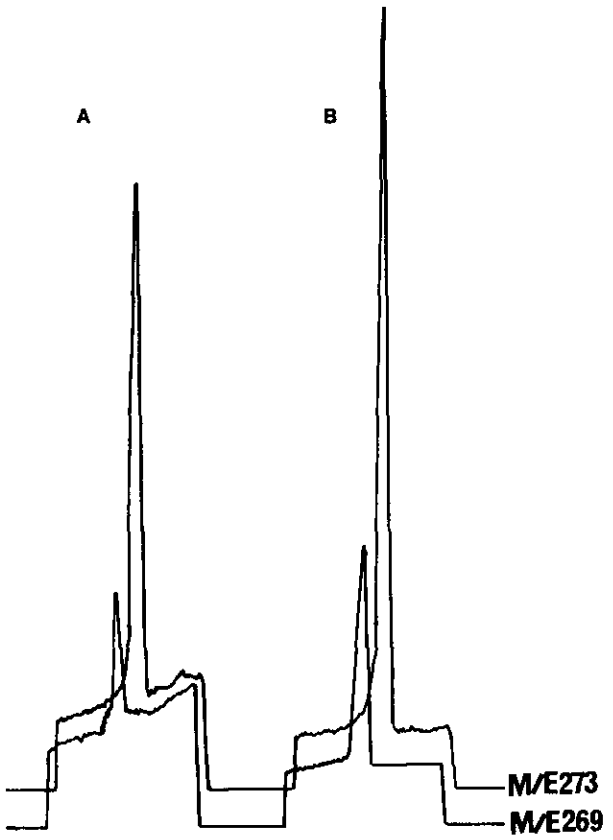


FIGURE 6. Ion chromatograms from the analysis of (A) brain and (B) blood from a rat sacrificed 60 min after receiving a 10 mg/kg dose of nordiazepam.

imine bond, since the σ values for *meta* and *para* fluoro substituents are 0.34 and 0.06, respectively (*20*). Thus, our study suggests that the more electron-deficient the imine bond, the higher the reaction rate of the compound with the thermal electrons in the source or the lower the rate of the reverse autodetachment reaction, i.e. the more stable the generated anion.

We were hoping that a brain tissue assay for nordiazepam could be developed by using the NCI technique. This compound is a key product in the metabolism of Valium, Nobrium, Tranxene, Ativan, and Verstran and has itself been used as a hypnotic agent (*21*). Attempts to analyze this compound using PCI with a simple sample workup were unvariably frustrated by the presence of interfering substances, especially lipids, in the biological extracts. The results using NCI, on the other hand, were quite satisfying.

The methane NCI mass spectra of nordiazepam and nordiazepam-d_2, the as internal standard in the assay, can be seen in Figure 3. As expected, the spectra consist principally of each compounds $(M\text{-}H)^-$ ion; *m/e* 269 and *m/e* 271 for nordiazepam and nordiazepam-d_2, respectively. For the nordiazepam response, *m/e* 269 was monitored in the GC effluent. Because of interference from nordiazepam's ^{37}Cl isotope peak, the ^{37}Cl isotope peak of nordiazepam-d_2 at *m/e* 273 was monitored in the assay for the internal standard response.

Typical ion chromatograms from the assay are shown in Figure 4. Ion chromatograms A and B were from the analysis of 0.3 g of control rat brain and 1 ml of control rat blood, respectively, each spiked with 1 ng of nordiazepam and 10 ng of nordiazepam-d_2. Both chromatograms were obtained from the injection of 1 out of the available 100 μl. Thus the response at *m/e* 269 represents approximately 10 pg injected on column. From the injection of many standard solutions over a period of several months, we estimate that the methane NCI sensitivity of nordiazepam is 15-25 times the compounds' methane PCI sensitivity.

Ion chromatogram A is remarkable. It illustrates that the sensitivity and specificity of the nci technique will permit the determination of low concentrations of a compound with intrinsic electron capture properties, e.g. nordiazepam, in a complex matrix like brain tissue with relatively simple sample workup.

A typical standard curve from the assay is shown in Figure 5. Because of nordiazepam's small response at *m/e* 273, the mass of the ion being monitored for the internal standard, the curve is nonlinear (*23*). As is our practice in such cases (*15, 16*), the standard curve was analyzed by a nonlinear least-squares fitting of the ion ratio versus amount added data to the isotope dilution equation $R = (x + A)/(Bx + C)$. In this equation R is the *m/e* 269 to *m/e* 273 ion ratio and x is the amount added. For the curve shown in Figure 5, the calculated constants (± SD) were $A = 3.8 \pm 2.2$ ng, $B = 0.13 \pm 0.02$, and $C = 54.8 \pm 4.5$ ng. An unknown amount *(x)* can be determined from an ion ratio *(R)* by using the rearranged equation $x = (RC - A)/(1 - RB)$.

Ion chromatograms from the analysis of the brain homogenate (A) and blood (B) from a rat

Table 3. Brain and blood concentrations of nordiazepam in three rats sacrificed at various times after receiving a 10 mg/kg oral dose of nordiazepam.

Time after dose, min	Blood concentration, ng/ml	Brain concentration, ng/g
20	45	147
40	32	66
60	13	29

sacrificed 60 min after receiving a 10 mg/kg oral dose of nordiazepam are shown in Figure 6. For both determinations, 1 of 100 μl were injected. The measured blood and brain homogenate concentrations were 13 and 11 ng/ml, respectively.

Brain and blood concentrations of nordiazepam in rats sacrificed at 20, 40 and 60 minutes after receiving a 10 mg/kg oral dose of nordiazepam are shown in Table 3. The mean (± SD) brain to blood concentration, equating 1 g of brain tissue to 1 ml of blood, was 2.52 ± 0.66.

The significance of this work can be simply stated. (1) In general, the methane mass spectra of compounds with the 1,3-dihydro-5-phenyl-1,4 benzodiazepin-2-one structure consist of only $(M-H)^-$ and M^- ions. The position of the H lost to generate the $(M-H)^-$ ion is unknown, although many possibilities have been eliminated. (2) A correlation of the relative sensitivities of a series of 1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-ones with structural features suggests that electron capture takes place at the 4,5 imine bond. (3) Because of the unique sensitivity and specificity of NCI, nordiazepam, an important metabolite of many of the clinically used benzodiazepines, can be determined in rat brain and blood with relatively simple sample workup.

The authors would like to acknowledge many helpful conversations concerning NCI instrumentation with Dr. G. Stafford, Finnigan Corporation. Dr. S. Markey, National Institutes of Health, suggested the use of magnets above the ion source and electron multiplier.

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