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DETERMINATION OF CLONAZEPAM AND ITS 7-AMINO METABOLITE IN PLASMA AND BLOOD BY GAS CHROMATOGRAPHY–CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A sensitive gas chromatography, ammonia chemical ionization mass spectrometry, ^{15}N isotope dilution assay has been developed to measure clonazepam and its 7-amino metabolite in blood or plasma. The method was used to measure both compounds in the blood of one subject administered a single 2-mg dose of clonazepam, and in the plasma of thirteen subjects on a clonazepam oral dosing regimen.

INTRODUCTION

Clonazepam (I, Table I) is a newly marketed antiepileptic agent (Clonopin®), which is useful in controlling "petit mal" minor motor seizures[1–5]. The principal metabolite of the drug is reported to be its nitro bioreduction product, II[6,7].

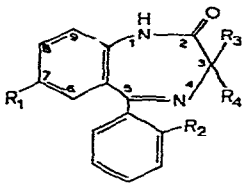
The development of a method for the determination of clonazepam and/or its metabolites in plasma is an analytical challenge. The method must be sensitive since the low therapeutic dose and large volume of distribution of clonazepam result in relatively low single dose and steady-state plasma levels[8,9]. In addition, the assay must possess great specificity because of the multiple drug nature of anticonvulsant therapy[10], and because of the extensive metabolism[6,7] of clonazepam.

Only one procedure[11], whose feasibility has been questioned[12], is available for measuring II, although several electron capture gas chromatographic (GC) assays for the determination of intact clonazepam have been reported[11,13,14]. An assay based on the acid hydrolysis of clonazepam and/or II to substituted benzophenones[12,15] is attractive because of the excellent GC and electron capturing properties of the generated benzophenones. The data from such a method, however, could be misleading since III, a known metabolite of clonazepam, would give the same benzophenone as clonazepam. In addition, two other clonazepam metabolites, IV and V, would yield the same benzophenone as II.

The purpose of the present investigation was to develop a simple, specific and relatively rapid method for the simultaneous determination of clonazepam and II. Such an assay would be useful in answering some of the basic research questions

TABLE I

CHEMICAL STRUCTURES AND THEIR DESIGNATION



Compound	R_1	R_2	R_3	R_4	*MH⁺ (principal isotopic species)*
I	NO_2	Cl	H	H	*m/e* 316
II	NH_2	Cl	H	H	*m/e* 286
III	NO_2	Cl	OH	H	*m/e* 332
IV	NH_2	Cl	OH	H	*m/e* 302
V	$NHCCH_3$ (O)	Cl	H	H	*m/e* 328
^{15}N-I	$^{15}NO_2$	Cl	H	H	*m/e* 317
^{15}N-II	$^{15}NH_2$	Cl	H	H	*m/e* 287
VI	NO_2	H	H	H	*m/e* 282
VII	NH_2	H	H	H	*m/e* 252
$^{2}H_2$-I	NO_2	Cl	^{2}H	^{2}H	*m/e* 318

concerning the pharmacokinetics, drug interactions, and plasma concentration-anticonvulsant response relationships for both clonazepam and/or its amino metabolite.

The assay developed, which is outlined in Fig. 1, features a simple extraction of plasma or blood, followed by GC–mass spectrometry (MS) analysis of a portion of the residue remaining after evaporation of the extraction solvent. A high degree of specificity is obtained by monitoring the MH^+ ions of clonazepam and its 7-amino metabolite, generated by ammonia chemical ionization (CI). Assay accuracy is insured by the use of stable isotope analogs of clonazepam and II as internal standards. The limit of detection of the method, 1 ng/ml for clonazepam and 2 ng/ml for II, is sufficient for measuring clonazepam and its metabolite in most, if not all, subjects on a chronic clonazepam dosing regimen, and in many subjects following administration of a single dose of clonazepam.

EXPERIMENTAL

Materials

Benzene and 1,2-dichloroethane were of analytical grade (Fisher Scientific, Springfield, N.J., U.S.A.), while the ethyl acetate, methanol, dichloromethane and hexane were of nanograde quality (Mallinckrodt, St. Louis, Mo., U.S.A.). Triethylamine and anhydrous dimethylformamide were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). Methanol solutions (1 mg/ml) of I and II were diluted with 0.01 *M* tris buffer (pH 7.4, Sigma, St. Louis, Mo., U.S.A.) to give solutions containing either 50 ng/ml (solution A) or 25 ng/ml (solution B) of both compounds.

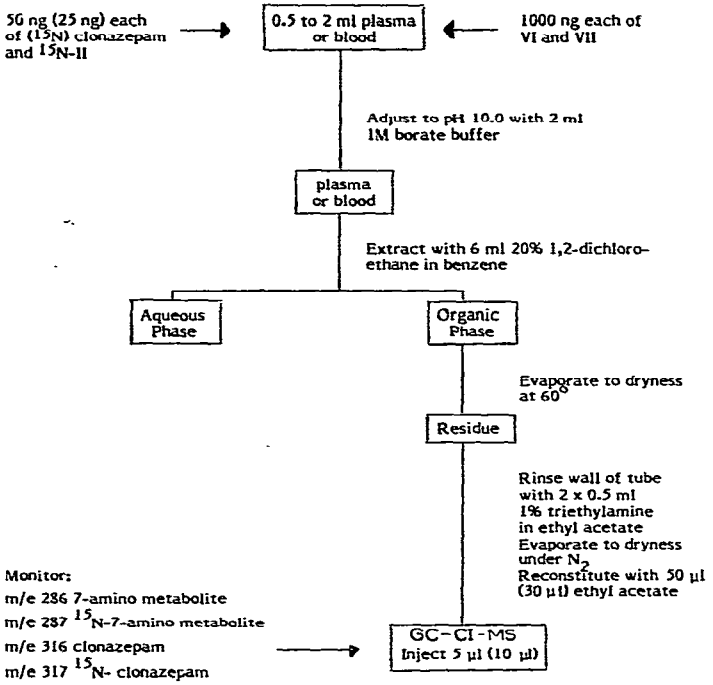


Fig. 1. Assay procedure for the determination of clonazepam and its amino metabolite in plasma from a subject on a steady-state clonazepam dosing regimen. The quantities in parentheses are those used to determine these compounds in subjects who have received a single dose of clonazepam.

Solutions containing ^{15}N-I and ^{15}N-II (50 ng/ml, solution C and 25 ng/ml, solution D) were prepared in the same manner. Compounds VI and VII were dissolved in methanol to give a solution containing 10 µg/ml of both compounds (solution E). Borate buffer (pH 10.0) was prepared as previously described[16]. Methane (ultra high purity), helium (high purity) and ammonia (anhydrous lecture bottle) were obtained from Matheson Gas Products (East Rutherford, N.J., U.S.A.). Culture tubes (16 ml) with Teflon®-lined screw caps (Pyrex 9825), and 5-ml centrifuge tubes (Pyrex 8061) were obtained from Scientific Glass (Palo Alto, Calif., U.S.A.). All glassware was cleaned with detergent, treated with a 1% aqueous solution of Siliclad® (Clay-Adams, Parsippany, N.J., U.S.A.), and dried.

^{15}N-I was prepared according to a reported procedure[17] with $K^{15}NO_3$ (99% ^{15}N, Koch Isotopes, Cambridge, Mass, U.S.A.) serving as the source of the nitrating agent. ^{15}N-II was prepared by the hydrogenation of ^{15}N-I in the presence of Raney nickel[17]. Both compounds had satisfactory IR, UV, proton magnetic resonance (PMR), and low resolution electron ionization (EI) mass spectral properties. High resolution, precise mass determinations of the EI generated M^+ · ions for both compounds were within 1 millimass of their theoretical values.

$^2H_2$-I was prepared by refluxing clonazepam (100 mg), deuterium oxide (1 ml, 99% ^2H_2O, Stohler Isotopes, Waltham, Mass., U.S.A.), and anhydrous redistilled dimethylformamide (8 ml) for 4 days at 100°[18]. After cooling the solution was diluted with water (20 ml) and extracted 3 times with diethyl ether. The combined ether extracts

were washed 3 times with water (20 ml), the ether removed, and the residue recrystallized from methylene dichloride–hexane. This compound, from a comparison of the EI-MS of $^2H_2$-I and I, was found to be 4.5% monodeuterated and 94.4% dideuterated. A comparison of the PMR spectra of $^2H_2$-I and I showed the deuterium to be located exclusively on carbon 3.

Instrumentation

A Finnigan 1015 or Finnigan 3200 mass spectrometer was used in conjunction with the manufacturer's model 9500 gas chromatograph, and either a Finnigan model 6000 data system or Finnigan Promim® peak monitor. The glass column (1.0 m × 2 mm I.D.) was packed with 1% OV-25 on 100–120 mesh Gas-Chrom Q (Applied Science Labs.), and conditioned with no flow for 2.5 h at 300° and with a 60-ml/min helium flow overnight at 280°. The operating conditions were column temperature 260°, injection port temperature 265°, carrier gas (methane) pressure 20 p.s.i. Ammonia was introduced via the direct insertion probe gas inlet. The ammonia ion source gas pressure was 0.2 torr, with a combined methane and ammonia ion source pressure of 1.5 torr. The typical ion source operating conditions were: ion energy, +2.8 V; ion repeller, +0 V; lens, −25 V; electron energy, −150 V; filament emission, 1 mA. The voltage across the continuous dynode electron multiplier was −2,000 V. The ions monitored were *m/e* 286 (MH^+ of II), *m/e* 287 (MH^+ of ^{15}N-II), *m/e* 316 (MH^+ of I), and *m/e* 317 (MH^+ of ^{15}N-I).

Assay procedure

Steady-state plasma concentrations. One or 0.5 ml of plasma or blood was transferred into a 16-ml culture tube. To the tube was added solution C (1 ml), solution E (0.1 ml) and pH 10 borate buffer (2 ml). The mixture was vortexed (Vortex Genie, Scientific Industries), 6 ml of 20% 1,2-dichloroethane in benzene was added, and the sample was extracted by gentle shaking for 15 min on a variable-speed reciprocating shaker (Eberbach). The tube was centrifuged (Damon Model CRU-500) at 10° for 10 min at 1500 *g*. The organic phase was transferred by a Pasteur pipet to a 5-ml centrifuge tube, and the solvent was removed at 60° with a gentle stream of nitrogen (N-Evap., Organomation Assoc.). The residue was concentrated into the tip of the centrifuge tube using two 0.5-ml portions of 1% triethylamine in ethyl acetate. The solvent was removed and the dry residue was redissolved in 50 μl of ethyl acetate. A 5-μl volume of this solution was injected into the gas chromatograph–mass spectrometer. Approximately 45 sec after sample injection, the GC divert valve was closed to allow the GC effluent to enter the ion source, and the ion source supplies were turned on 15 sec later. Selected ion recording was initiated 60 sec after injection. Under the GC conditions used, I and ^{15}N-I eluted at approximately 75 sec after sample injection, while II and ^{15}N-II eluted at approximately 90 sec after sample injection.

The concentrations of I and II in blood or plasma were determined from measurements of the peak heights of the selected ion current signals. The peak height ratios for *m/e* 286 *vs. m/e* 287 and *m/e* 316 *vs. m/e* 317 were calculated and converted to compound amounts using standard curves. The standard curves were prepared from analyses of the ion ratio *versus* compound amount data obtained from specimen control samples spiked with 0, 25, 50, 75 and 100 ng each of I and II. The data for

the standards were fitted by a non-linear least squares computer program to the equation

$$y = \frac{x + A}{Bx + C}$$

where y is the ratio of *m/e* 316 to *m/e* 317 or *m/e* 286 to *m/e* 287, x is the corresponding amount (ng) of unlabelled material added, and A, B, and C are constants. Once found, the values for A, B and C are used in one of two ways to calculate an unknown amount of clonazepam or II from an experimentally determined ion ratio. Either they can be used directly in the above equation, or they can be used to draw a calculated curve of amount *versus* ion ratio. The actual concentrations of I and II are found by dividing the amount of each compound determined by the volume of blood or plasma used for the assay.

Single-dose plasma concentrations. The procedure used for single-dose clonazepam plasma concentrations was identical to that used for steady-state plasma assays with the following exceptions. Either 1 or 2 ml of plasma was used for extraction. Solution D instead of C was added to the plasma. The final residue was reconstituted in 30 μl of ethyl acetate and 10 μl of this solution was injected into the GC–MS instrument. The standard curves were prepared using 0, 10, 20, 30, and 50 ng of added I and II (solution B).

RESULTS AND DISCUSSION

As expected[19], the ammonia CI mass spectra of clonazepam, its 7-amino metabolite, and their ^{15}N stable isotope analogs consist principally of each compound's MH^+ molecular ions (Fig. 2). Typical ion chromatograms from the monitoring in the GC effluent the MH^+ ions of I, II, and their ^{15}N analogs following injection of a portion of the extract from a patient sample can be seen in Fig. 3. Unfortunately, the ion chromatogram for *m/e* 286 contains a fragment ion ($MH^+ - 30$) from clonazepam in addition to the MH^+ ion of II. Thus the GC column used for the assay must be able to resolve clonazepam and II. In this regard, we found OV-25 to be superior to OV-17 as the GC liquid phase.

The nature of the process responsible for the generation of the $MH^+ - 30$ ion in the ammonia–methane mass spectra of clonazepam was not immediately apparent. The generation of this ion is not an artifact of using ammonia as a reagent gas, since it also occurs when either methane (Fig. 4B) or isobutane (Fig. 4D) is used. Nor is it an thermal artifact caused by GC, since it also occurs when the clonazepam spectrum is obtained using the direct insertion probe (Figs. 4C and 4D).

Three possibilities for the formation of this ion were considered: $MH^+ - NO$, $MH^+ - CH_2O$, and reduction of the nitro group to an amine by the ionizing plasma in the ion source. Unfortunately the equipment required to differentiate between these possibilities by a precise mass determination under CI conditions was not available. Nevertheless, the first possibility is eliminated by the spectrum of ^{15}N-I which shows no loss of ^{15}NO (Fig. 2B). The second possibility would be analogous to the $M^+ \cdot - 29$ ion in the EI mass spectra of similar benzodiazepines[20–23]. Labelling experiments have shown this process in the EI spectra to involve the carbonyl and one hydrogen from

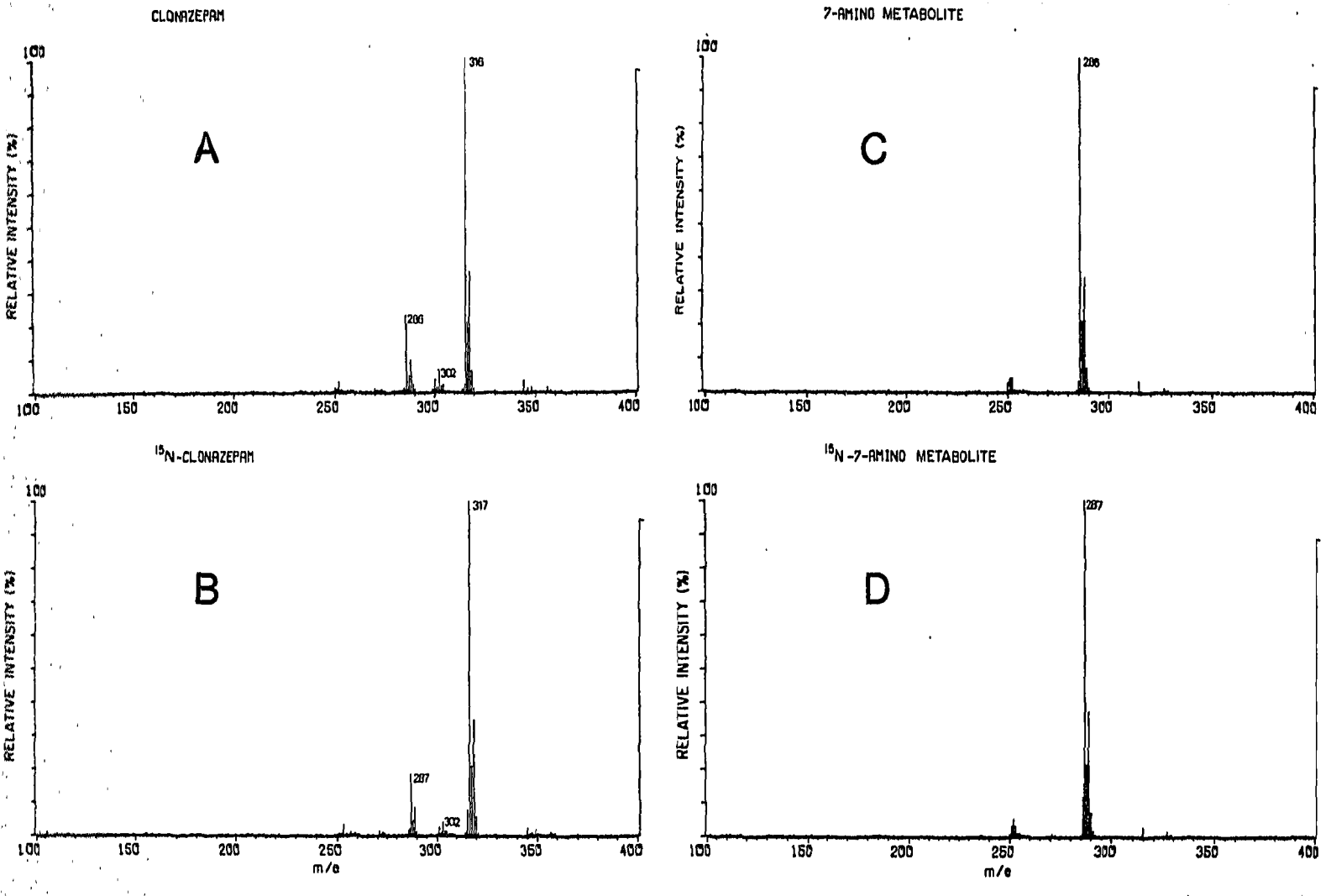


Fig. 2. Ammonia chemical ionization mass spectra of (A) clonazepam, (B) ^{15}N-I, (C) II and (D) ^{15}N-II. Spectra were obtained under the GC–MS conditions described in the text (Experimental).

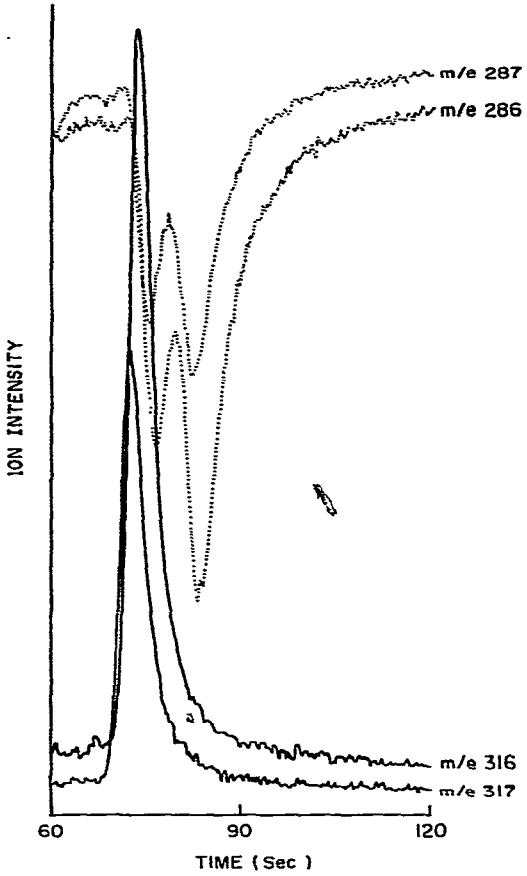


Fig. 3. Ion chromatograms from the analysis of 0.5 ml plasma from a subject on a steady-state clonazepam dosing regimen. The ions at *m/e* 287 and *m/e* 317 represent 25 ng each ^{15}N-I and ^{15}N-II. 0.5 μl out of the available 30 μl were injected into the GC–MS instrument. The concentrations of clonazepam and its amino metabolite were determined to be 105 and 139 ng/ml, respectively. The data was obtained using the Finnigan Promim® peak monitor.

carbon 3[24]. Compound $^2H_2$-I was synthesized to determine if a similar process is responsible for the $MH^+ - 30$ ion in the CI spectrum. The ammonia CI mass spectrum of this compound showed considerable deuterium exchange in the ionizing plasma (Fig. 4E). The deuterium incorporation by EI mass spectral analysis was 94.4% $^2H_2$ and 4.5% $^2H_1$, while the incorporation by ammonia CI mass spectral analysis was 41.1% $^2H_2$, 40.2% $^2H_1$, and 18.8% $^2H_0$. Nevertheless the CI generated $MH^+ - 30$ ion has almost the same deuterium incorporation, 44.8% $^2H_2$, 37.4% $^2H_1$, and 17.7% $^2H_0$ as the MH^+ ion. Thus, this data does not support CH_2O as the elements lost in the generation of the $MH^+ - 30$ ion. The third possibility, reduction of the nitro group to the amine in the ionizing plasma, is consistent with all the available data. The analogous synthetic reduction of a nitro group is often accomplished with mineral acid in the presence of a metal catalyst[25]. Further evidence for this mechanism can be inferred by the existence of the weak ions at *m/e* 300 and *m/e*

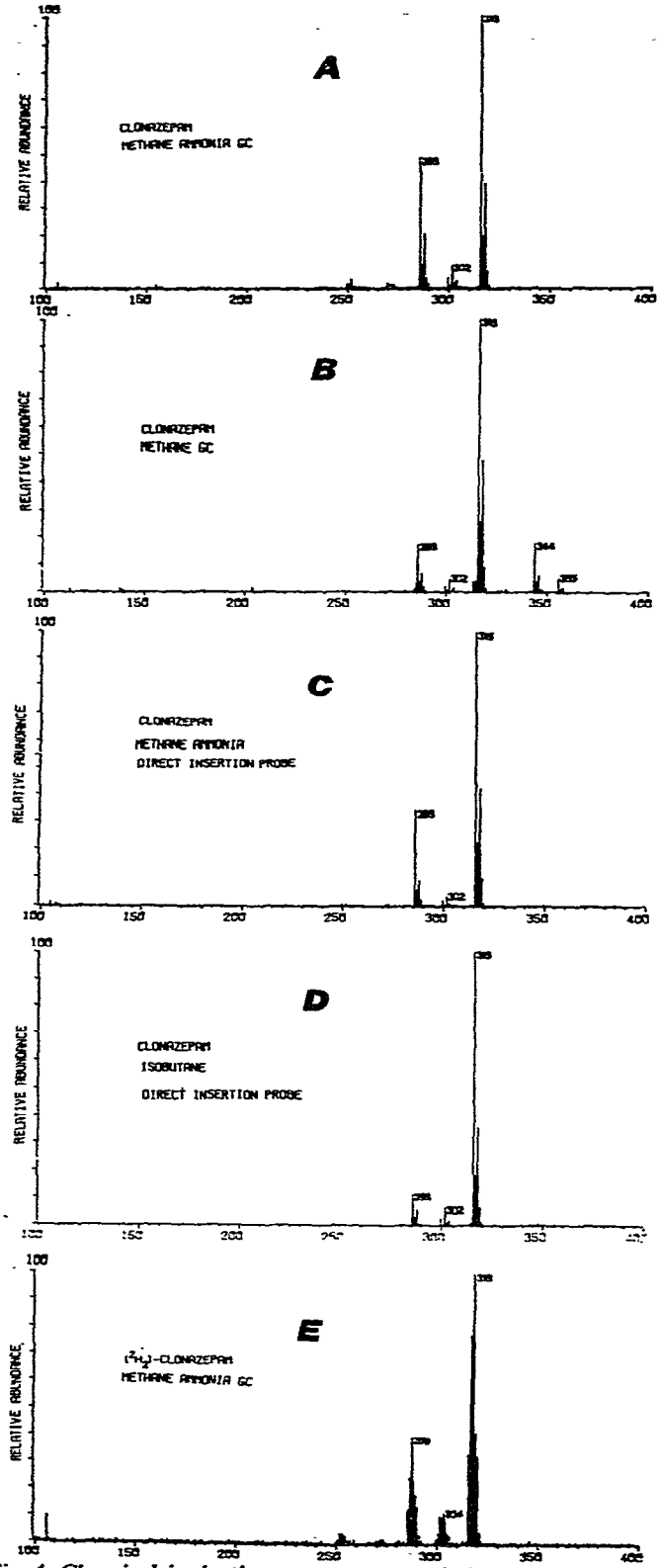


Fig. 4. Chemical ionization mass spectra of clonazepam, I. (A) ammonia, GC inlet; (B) methane, GC inlet; (C) ammonia, direct insertion probe; (D) isobutane, direct insertion probe; and (E) $^2H_2$-I, ammonia, GC inlet.

302 present in the isobutane, methane and methane–ammonia spectra (Figs. 2 and 4). These ions would be the nitroso and hydroxylamine species, which are intermediates in the reduction of a nitro function to an amine.

Human control plasma subjected to the assay procedure shows no significant background ions at *m/e* 286, 287, 316, and 317. This is quite remarkable considering the simplicity of the extraction procedure and the polarity of the extraction solvent used.

Compound losses during the assay procedure were found to be quite significant unless special precautions were taken. Unfortunately, the ^{15}N stable isotope analog used gives only a modest increase in mass over the substances to be determined. Because of this, the use of a large ratio of stable isotope analog to substance being measured is not feasible. Recourse was made, therefore, to carrier substances (compounds VI and VII), special treatment of the glassware (Siliclad®), and the addition of small amounts of triethylamine to the solvent used to wash down the glassware, in order to avoid unacceptably low assay recoveries.

Assay recoveries from plasma were determined from ten samples prepared to supply standard curve data. The recoveries ±standard deviation (S.D.), determined from a comparison of the ion peak heights obtained from the injection of known amounts of the ^{15}N internal standards versus the ion peak heights obtained from the ^{15}N internal standards carried through the assay, were 114.5 ± 15.3% for clonazepam and 79.6 ± 10.0% for the 7-amino metabolite. Recoveries from blood were too erratic to be quoted. The stable isotope analogs, however, enable the method to be used with blood.

A typical standard curve relating the amount of unlabelled clonazepam and II added to plasma to the resulting peak height ratios can be seen in Fig. 5. As expected from a theoretical analysis, the curve is not a straight line[26–28]. The equation for a constant value of (^{15}N) clonazepam, C, with varying amounts of clonazepam, x, is:

$$y = \frac{x + A}{Bx + C}$$

where y is the ratio of *m/e* 316 to *m/e* 317, B is the ratio of the *m/e* 317 to *m/e* 316 ions in clonazepam and A is equal to C times the ratio of the *m/e* 316 to *m/e* 317 ions in ^{15}N-I. The above definitions are valid only for 100% ^{15}N label in the internal standard and identical peak ratios in the ion clusters of the ^{14}N and ^{15}N compounds. A non-linear least square analysis of the experimental standard curve data for clonazepam in Fig. 5 gives a value ± S.D. of A = 2.8 ± 0.7 ng, B = 0.24 ± 0.02 and C = 43.4 ± 1.9 ng. The predicted values for A, B and C, based on the separate injection of ^{14}N and ^{15}N standard samples, would be 3.5 ng, 0.17 and 50 ng, respectively. An analysis of the amino metabolite standard curve gives similar results.

The limit of detection of the assay is a function of the condition of the GC column, and the cleanliness of the ion source and quadrupole rods. A typical GC column must be conditioned with multiple injections of blank plasma extracts before maximum sensitivity can be obtained. After several hundred to a thousand injections, the column will usually have to be replaced. For the same GC column, ion source, and quadrupoles, the assay sensitivity was invariably twice as good if the Promin®

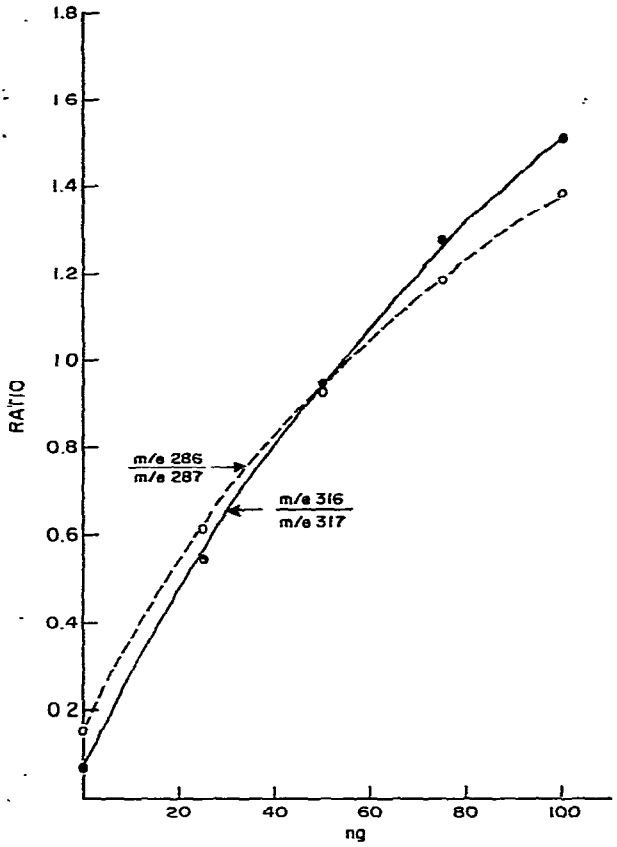


Fig. 5. Standard curves for the addition of 0, 25, 50, 75 and 100 ng of both clonazepam and II to plasma. 50 ng each of ^{15}N-I and ^{15}N-II were used as internal standards.

rather than model 6000 data system was used to collect the data. The injection of 1 μl of a solution containing 1.0 ng/μl of I, II, ^{15}N-I, and ^{15}N-II typically gives a peak with a signal-to-noise ratio greater than 10:1 for each of the MH^+ ions.

To evaluate the reproducibility of the method, six separate 1-ml plasma samples and six 1-ml blood samples were spiked with 50 ng each of clonazepam and its amino metabolite, and analyzed by the procedure described for steady-state clonazepam levels. The results (Table II) suggest an average combined blood and plasma accuracy of 4.8% and precision of 3.7%.

The concentration of clonazepam and its amino metabolite in plasma samples from thirteen individuals who were on a 1–15-mg/day clonazepam oral dosing regimen can be found in Table III. The values, which are similar to that previously reported by Sjo *et al.*[29], range from 10 to 122 ng/ml for clonazepam and from 18 to 148 ng/ml for the amino metabolite. For these subjects the ratio of amino metabolite to clonazepam $\pm$ S.D. was 1.38 $\pm$ 0.46.

The results of the analyses of several blood samples from a subject who had received a single 2-mg dose of clonazepam can be seen in Fig. 6. The concentration of clonazepam could be followed for 48 h following dosing. The peak clonazepam

TABLE II

ASSAY ACCURACY AND REPRODUCIBILITY OF THE ASSAY PROCEDURE

Six 1 ml blood samples and six 1-ml plasma samples, each spiked with 50.0 ng of clonazepam and 50.0 ng of its amino metabolite, II, were analyzed. The data were obtained using a Finnigan Model 6000 Data System.

	Concentration (ng/ml)			
	Blood		*Plasma*	
	Clonazepam	*Amino metabolite*	*Clonazepam*	*Amino metabolite*
	49.4	52.3	57.1	52.5
	49.6	50.0	55.3	47.8
	52.1	51.9	54.5	52.7
	52.9	53.6	55.4	52.3
	48.9	50.0	55.8	48.1
	51.9	53.4	54.1	56.1
Mean ± S.D.	50.6 ± 3.8	51.8 ± 3.1	55.4 ± 1.1	51.6 ± 3.1

TABLE III

PLASMA CONCENTRATIONS OF CLONAZEPAM AND ITS AMINO METABOLITE IN PATIENTS ON A DAILY CLONAZEPAM DOSING REGIMEN

All the patients were receiving various amounts of other anticonvulsants in addition to clonazepam.

Patient	*Sex*	*Age*	*Weight (kg)*	*Daily dose clonazepam (mg/day)*	*Concentration (ng/ml)*	
					Clonazepam	*Amino metabolite*
W.H.	M	3	18.1	12	68	70
S.D.	F	16	51.7	5	20	18
H.G.	F	5	18.5	5	10	20
D.S.	M	13	58.5	3	11	22
C.J.	M	8	29.9	4.5	72	89
J.B.	M	4	24.4	1	72	89
C.J.	M	7	27.3	4.5	112	145
J.B.	M	4	22.3	5	35	43
L.B.	F	17	45.5	3	24	32
L.B.	F	19	45.0	3	26	55
T.H.	F	9	20	15	122	148
N.R.	M	16	57.7	10	56	79
C.R.	F	15	63	6	48	108

blood concentrations of 13.6 ng/ml for this subject was attained 2 h after dosing. A drug half-life of 31 h was obtained using the blood concentration data from the 8–48-h post-dose samples. This clonazepam half-life falls in the range previously reported by Berlin and Dahlstrom (19–60 h)[30] and Kaplan *et al.* (19–39 h)[31].

The assay described, although useful in many applications, must be further developed in order to make it a suitable analytical tool for all phases of clonazepam research. The assay sensitivity should be increased by an order of magnitude to enable concentration determinations to be made over a period of several clonazepam

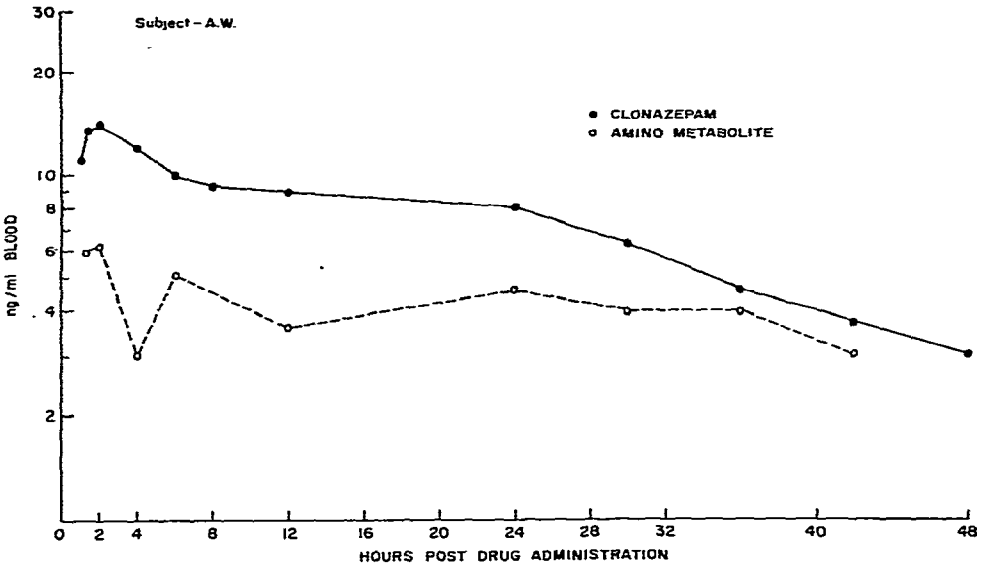


Fig. 6. Blood concentration–time curves for a subject who had received a 2 mg-oral dose of clonazepam.

half-lives following single-dose administration, and to decrease the amount of blood or plasma required by the method. In this regard, we are currently investigating the utility of negative ion CI for the analysis of clonazepam[32]. In addition stable isotope analogs whose masses are 5 a.m.u. or more greater than clonazepam or II would be desirable, in order to permit the use of a greater ratio of labelled to unlabelled material. The syntheses of such standards and the expansion of the assay to measure other clonazepam metabolites are currently under investigation.

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