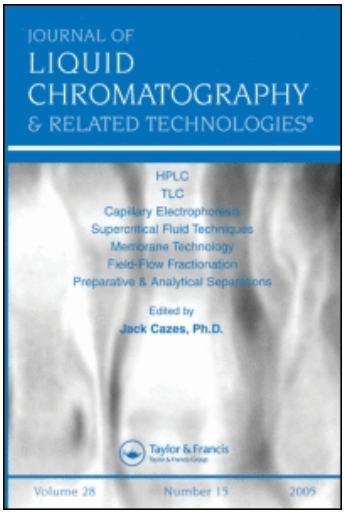

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Separation of Clonazepam and Five Metabolites by Reverse Phase HPLC and Quantitation from Rat Liver Microsomal Incubations

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SEPARATION OF CLONAZEPAM AND FIVE METABOLITES BY REVERSE PHASE HPLC AND QUANTITATION FROM RAT LIVER MICROSOMAL INCUBATIONS

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ABSTRACT

Clonazepam, its nitro-reduced metabolites, and the 3-hydroxylated derivatives, were separated by reverse-phase hplc using a four solvent optimization procudure. The separation was used to quantitate the metabolism of ^{3}H-clonazepam in rat liver microsomes under aerobic or anaerobic conditions by collection of HPLC eluents and liquid scintillation counting. The predominant metabolite in aerobic incubations was 3-hydroxyclonazepam. The primary anaerobic metabolite was 7-aminoclonazepam. The identity of the microsomal metabolites was confirmed by chemical ionization mass spectrometry. This separation could be applicable for future metabolic studies of clonazepam.

INTRODUCTION

Clonazepam (Figure 1), a benzodiazepine whose structure contains a nitro-group, is an anticonvulsant effective in the treatment of Lennox-Gastaut syndrome, akinetic and myoclonic seizures, petit mal seizures not controlled by succinimides, and status epilepticus (1). The only comprehensive study on the metabolism of the compound in rat, dog, and man found that in man, the major metabolite was 7-aminoclonazepam, as well as small amounts of the 7-acetamido,

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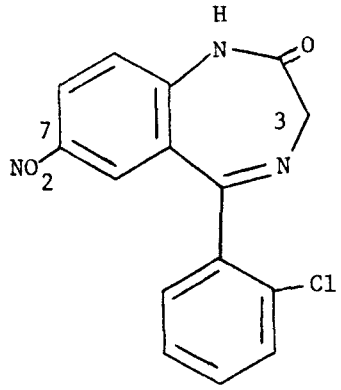

FIGURE 1. Structure of Clonazepam

3-hydroxy-7-amino, and 3-hydroxyamino, and 3-hydroxy-7-acetamido metabolites in the urine. Also identified were β-glucuronidase/ sulfatase hydrolyzable conjugates of 7-aminoclonazepam and a 7-amino-phenol derivative. The rat had a metabolic pattern similar to man but, additionally, 7-acetamidoclonazepam was found as a major free metabolite in the urine. A conjugated phenolic derivative and conjugated 7-aminoclonazepam were also major urinary metabolites in the rat. Small quantities of metabolites hydroxylated in the 3-position were observed. In contrast to man and the rat, the major metabolites in the dog were 3-hydroxy-7-aminoclonazepam and 7-aminoclonazepam, and a conjugate of 3-hydroxyclonazepam. The metabolites were identified by thin layer chromatography, although levels were not quantitated in this study. Subsequent to this report, studies have focused on levels of the parent compound alone, or together with the 7-amino or 7-acetamido metabolites, either by TLC (3), HPLC (4), GC (5-7), or GC-MS (8-10). This paper reports on the analysis of clonazepam, its reduced metabolites, and the 3-hydroxyl derivatives by reverse phase HPLC combined with liquid scintillation counting of eluants. Metabolism of ^{3}H-clonazepam was studied in rat liver microsomes under aero-

bic and anaerobic conditions. This is the first paper to quantitate the 3-hydroxyl metabolites of clonazepam.

MATERIALS AND METHODS

Authentic samples of flunitrazepam, ^{3}H-clonazepam, clonazepam, aminoclonazepam, 3-hydroxyclonazepam, and 3-hydroxy-aminoclonazepam were gifts from Hoffman-LaRoche, Nutley, N.J. Acetamidoclonazepam and 3-hydroxyacetamido clonazepam were made from the respective amino compounds by reaction with acetic anhydride. For example, 7-aminoclonazepam (250 mg) was dissolved in 10 ml of 5% HCl, neutralized with 5% NaOH until the solution was turbid, and 5% HCl was added dropwise until the solution cleared. One ml of acetic anhydride was added with a few pieces of ice and the mixture was stirred for five minutes. Sodium acetate trihydrate (2 ml of a 1g/2ml solution) was added and a flocculent off-white precipitate was obtained. After filtering and drying the yield was 80%, and product was greater than 98% pure by HPLC. The compound's identity was confirmed by mass spectrometry. ^{14}C-acetamidoclonazepam was synthesized as above by acetylation of 100 mg of cold aminoclonazepam with 250 µCi [1-^{14}C]-acetic anhydride (Amersham, Arlington Heights, Il) first, followed by addition of cold acetic anhydride to complete the reaction. The specific activity of the product was 0.6µCi/mg. ^{3}H-aminoclonazepam was prepared by reducing 100 mg of ^{3}H-clonazepam (specific activity 2.5µCi/mg) by hydrogenation over 5% Pd on charcoal in ethanol at 2 atmospheres of H_2. One equivalent of H_2 was used per equivalent of substrate. The product was 89% pure by HPLC, and was further purified by recrystallization from hot ethanol. The specific activity was 2.5µCi/mg.

Methyl alcohol, tetrahydrofuran, and acetonitrile were "distilled in glass," HPLC grade solvents from Burdick & Jackson, Muskegan, MI. Distilled water was filtered through a Milli-Q Reagent Grade Water System (Millipore Corp., Bedford, MA) and then used to make a 10 mM sodium phosphate buffer, pH 7.0. NADP, glucose-6-phos-

phate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chem. Co., St. Louis, MO.

Separations were accomplished at 45°C on a Zorbax C-8 column (4.6 mm ID x 25 cm; DuPont Instruments, Wilmington, DE) using a DuPont Instruments 850 Liquid Chromatograph with a temperature-controlled column compartment and a model 850 absorbance detector with a 254 nm filter. Two mobile phases were used. The first mobile phase (A solvent) was methanol/tetrahydrofuran/10 mM phosphate buffer, pH 7.0 (53/15/212) which ran for 9 minutes, followed by the B solvent which was methanol/acetonitrile/10 mM phosphate buffer, pH 7.0 (50/35/125) which ran for 15 minutes. The flow rate was 2.0 ml/min.

Separation of clonazpeam and its metabolites was achieved by performing optimization of solvent strength and selectivity using four solvents (11,12). Briefly, this technique employs solvents from three corners of the Snyder solvent selectivity triangle (13), namely methanol, acetonitrile, and tetrahydrofuran along with water (buffer) to adjust the solvent strength. An appropriate solvent strength is picked to give k values from 0.5 to 10, starting with a methanol/buffer mixture. After the appropriate solvent strength is selected, equal solvent strengths for acetonitrile and tetrahydrofuran-buffer mixtures are calculated. Seven isocratic experiments are done using the three solvents alone, 50:50 mixtures of solvents, and an equal mixture of the three solvents. Calculations of k values are performed and a mixture chosen that would give the best separation. Although a separation of all the compounds was achieved in a single solvent that would be adequate for UV detection, the separation was not applicable for peak collection for radioactivity determination because of the close proximity of the reduced metabolites to each other. Therefore, optimization was subsequently performed using a weaker solvent strength to further separate the most polar metabolites, followed by an optimized stronger solvent to elute 3-hydroxy-clonazepam and an internal standard, flunitrazepam.

Chemical ionization-mass spectrometry was utilized to identify metabolites from HPLC eluants. The HPLC eluant was collected in

glass scintillation vials and the organic components of the eluant were removed under a stream of nitrogen in a 65°C water bath. The remaining buffer (ca. 1 ml) was extracted with 1 ml of chloroform: ethyl acetate (1:1). The organic layer was removed, transferred to small vials and evaporated to dryness. For mass-spectrometry, the samples were redissolved in ethyl acetate and an appropriate aliquot was evaporated onto a porclain probe. The mass spectrometer was a VG-7070 (VG Analytical, Altrincham, England) operated in the chemical ionization mode with a source temperature of 240°, and methane as the reagent gas, to cause a thermal degradation of the 3-hydroxylated derivatives resulting in a net loss of H_2O. This thermal rearrangement has been characterized for oxazepam, a benzodiazepine with a hydroxyl group in the same position (14).

Rat liver microsomes were prepared from 175-225 g male, Sprague-Dawley rats (Tyler Labs, Bellevue, WA) maintained on water and Wayne Lab-Blox F-6 rat chow ad lib in the following manner. The rats were decapitated, and their livers excised, weighed, and homogenized with 3 volumes of .01 M, pH 7.4 isotonic KCl phosphate buffer per g liver. The homogenate was centrifuged at 9000 x g for 15 minutes and the supernatant was removed and centrifuged at 100,000 x g for 60 minutes on Model L2-65B Ultracentrifuge (Beckman Instruments). The pellet was washed by homogenizing with buffer followed by centrifugation at 100,000 x g for 60 minutes. After the second spin, the pellet was homogenized in buffer and protein content determined by the method of Lowry(15). Each 5 ml incubation contained 10 mg protein, 5 units of glucose-6-phosphate dehydrogenase, 0.5 mM NADP, 2 mM Mg^{++}, 4.6 mM glucose-6-phosphate, 46 mM Na phosphate (pH 7.4), and 0.5 mM ^{3}H-clonazepam (1μCi). Anaerobic incubations were performed in capped 30 ml vials with a rubber septum and connected in series by means of Tygon tubing attached to 18 g needles. Vials were alternately flushed with nitrogen, which was scrubbed with an Oxisorb cartridge (Supelco, Inc., Bellefonte, PA), and evacuated with a water-driven aspirator six times. A nitrogen flow of 5-10 cc per min. was maintained during the incubation. Aerobic incubations were done in open 30 ml vials. Incu-

bations were started by transferring the incubations from an ice bath to a 37°C Dubnoff metabolic shaker. After 15 minute incubation, metabolism was stopped by addition of 2 ml of acetone.

Incubations were extracted three times with a 10 ml volume of chloroform;ethyl acetate (1:1) after adjusting the pH to 9.5. The extracts were combined, filtered through a phase-separating filter (Whatman) to remove water, and evaporated to dryness under N_2. The samples were taken up in 200 µl of methanol and a 20 µl aliquot was injected on the HPLC. Aliquots of both the extract and the aqueous layer were counted by LSC on a Beckman LS-7500 liquid scintillation counter. CPM were automatically converted to DPM using a quench curve based on quenched flame-sealed standards (Beckman Instrument, Fullerton, CA). The extraction procedure removed 98.1 ± 1.7% of the total radioactivity and >95% of added ^{3}H-aminoclonazepam or ^{14}C-acetamidoclonazepam. HPLC peaks corresponding to authentic standards of the metabolites and clonazepam were collected and counted in 10 ml of Aquasol-2 (New England Nuclear, Boston, MA). Collections from boiled microsomal samples were used to determine background radioactivity.

RESULTS AND DISCUSSION

The HPLC separation profile of clonazepam and its metabolites is shown in Figure 2. This system was utilized to study the microsomal metabolism of clonazepam. A step-wise elution of the compounds using two solvents accomplished the goal of adequate separation for collection of metabolite fractions. Greater than 95% of the injected radioactivity was recoverable from the HPLC eluants.

The k values for the individual compounds in a single solvent are listed in Table I. Although the separation of the compounds was achieved in the optimized solvent system for UV detection, the separation was not adequate for collection of HPLC eluants, because the four most polar metabolites eluted too closely together. The table also shows data for flunitrazepam, which could be used as an in-

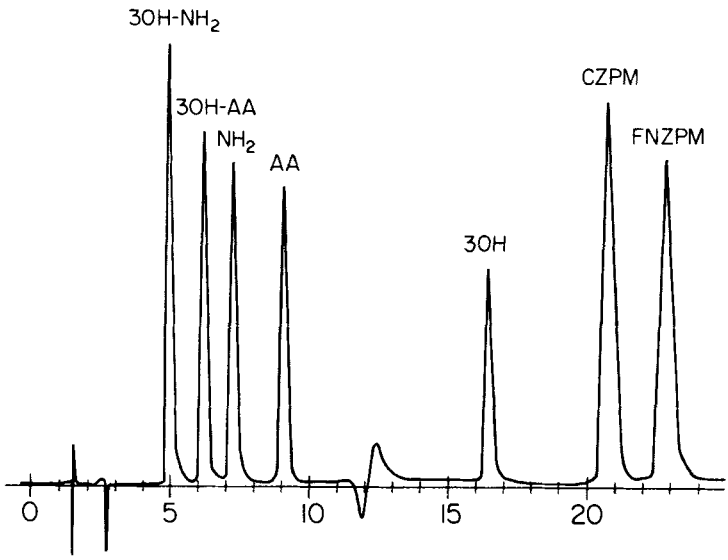

Figure 2. Separation of clonazepam and its metabolites by reverse phase HPLC.

The separation of clonazepam and its metabolites was achieved on a Zorbax C-8 column at a controlled temperature of 45°C. Two solvents were employed. The first solvent ran for nine minutes and was composed of methanol:tetrahydrofuran:10 mM phosphate buffer, pH 7.0 (53:15:212). The second solvent ran from 9 to 24 minutes and was composed of methanol:acetonitrile:10 mM phosphate buffer, pH 7.0 (50:35:125). The solvent change took about three minutes to reach the 254 nm UV detector. Abbreviations are 3OH-NH_2 = 3-hydroxyaminoclonazepam, 3OH-AA = 3-hydroxyacetamidoclonazepam, NH_2 = aminoclonazepam, AA = acetamindoclonazepam, 3OH - 3-hydroxyclonazepam, CZPM = clonazepam, and FNZPM = flunitrazepam. The scale is in minutes.

ternal standard if the sample was free of UV absorbing impurities. We estimate that the limit of sensitivity for clonazepam is 10 ng at 254 nm. The average plasma level is about 10 ng/ml in human patients (1), therefore, this assay would best be used to examine excreted levels of the drug and its metabolites.

The k values for the four polar metabolites at a weaker solvent strength are listed in Table II. Optimization at this solvent strength was used to accomplish the separation seen in Figure 2.

TABLE 1

The k values for clonazepam and metabolites.

Compound	k values in the solvent[a]					
	MeOH 50%	MeOH/THF 25%/15%	THF 30%	THF/AcCN 15%/17.5%	AcCN 35%	AcCN/MeOH 17.5%/25%
3OH-NH_2	0.75	0.81	1.09	0.92	1.10	1.13
3OH-AA	1.17	0.81	0.91	0.92	1.10	1.29
NH_2	1.21	1.08	1.54	1.54	1.48	1.71
AA	1.75	1.08	1.27	1.23	1.48	2.00
3OH	5.00	5.88	6.45	5.69	4.95	7.08
CZPM	7.25	7.46	8.00	8.38	8.62	12.08
FNZPM	8.24	8.81	9.91	8.92	12.33	15.08

[a] The balance of the solvent was 10 mM Na phosphate buffer, pH 7.0.

Separations were accomplished on a Zorbax C-8 column at a controlled temperature of 45°C and a flow rate of 2ml/min. Abbreviations are MeOH = methanol, THF = tetrahydrofuran, and AcCN = acetonitrile. For abbreviations of the compounds see Figure 2.

TABLE 2

The k values for the polar clonazepam metabolites in the optimization solvents at weaker solvent strengths.

Compound	k values in the solvent[a]						
	MeOH 34%	MeOH/THF 17%/7.5%	THF 15%	THF/AcCN 7.5%/10%	AcCN 20%	AcCN/MeOH 10%/17%	MeOH/THF[b] 18.9%/5.4%
3OH-NH_2	3.59	3.07	3.96	3.54	4.18	3.15	2.37
3OH-AA	6.33	4.89	3.67	3.40	4.18	4.85	3.21
NH_2	6.85	5.48	6.26	5.82	7.61	5.81	3.93
AA	11.33	8.23	5.85	4.63	7.14	8.11	5.21

[a] The balance of the solvent was 10 mM Na phosphate buffer, pH 7.0.

[b] k values in the optimized solvent (A solvent) of methanol:tetrahydrofuran:10 mM phosphate buffer, pH 7.0 (53:15:212).

Separations were accomplished on a Zorbax C-8 column at a controlled temperature of 45°C and at a flow rate of 2ml/min. Abbreviations are listed in Table 1 and Figure 2.

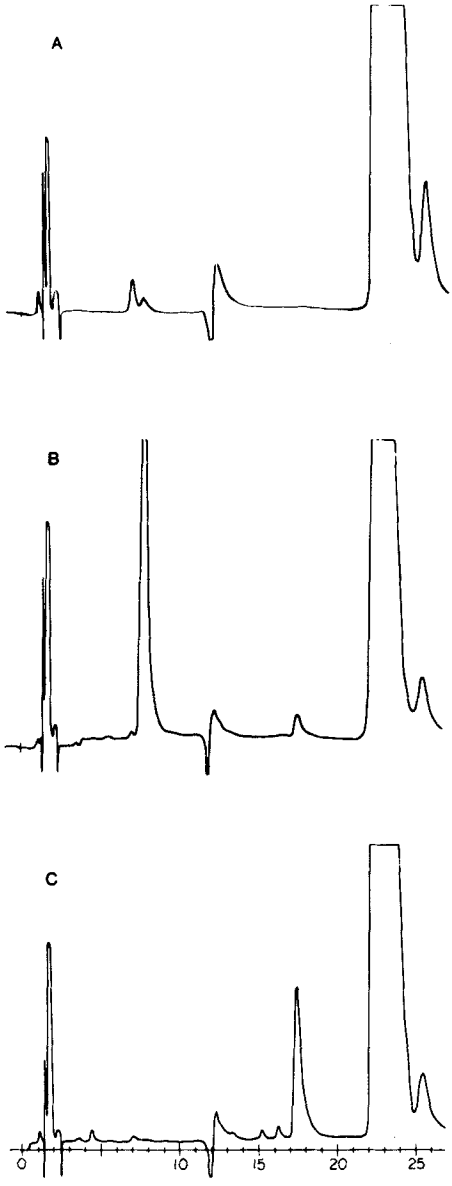

Figure 3. Aerobic and anaerobic microsomal metabolism of clonazepam.

HPLC separation of metabolites obtained from incubation of clonazepam with boiled microsomes (A), microsomes incubated in a nitrogen atmosphere (B), and microsomes incubated in air (C) obtained from rat liver.

Mixtures of tetrahydrofuran or acetonitrile with methanol both gave adequate separations, however, there was a significant tailing of the 3-hydroxy metabolites with acetonitrile. Therefore, a tetrahydrofuran/methanol optimized mobile phase was selected to separate the four most polar metabolites. This was subsequently followed by a second solvent with decreased polarity. Although 3-hydroxyclonazepam is easily separable from clonazepam, in microsomal extractions, clonazepam accounts for >90% of the radioactivity. Therefore, it was necessary to widely separate clonazepam from 3-hydroxyclonazepam. Experiments with boiled microsomes showed that reverse-tailing of the clonazepam peak was significant as much as 3 minutes before absorbance was observable on the UV detector. Methanol was chosen as the primary organic constituent in the "B solvent," because of cost. Acetonitrile was also added as a modifier to increase the separation of the internal standard, flunitrazepam, from clonazepam (see Table 1). The background levels (obtained from boiled mixtures) were <100 DPM/ml solvent in the "A" solvent range, and about 300 DPM/ml in the B solvent (eluent collected with retention time corresponding to the 3-hydroxyclonazepam peak) out of the total 150,000 DPM injected.

Figure 3 shows representative analyses of 15 minute microsomal incubations of clonazepam under aerobic and anaerobic conditions. Under aerobic conditions, 338.7 $\pm$ 29.6 pmoles/mg protein/min (n=4) of 3-hydroxyclonazepam were produced, which was identified by co-chromatography with authentic standard and by chemical ionization mass spectrometry by direct probe of HPLC eluant extracts. Figure 4 shows the mass spectrum of the HPLC eluant and the authentic 3-

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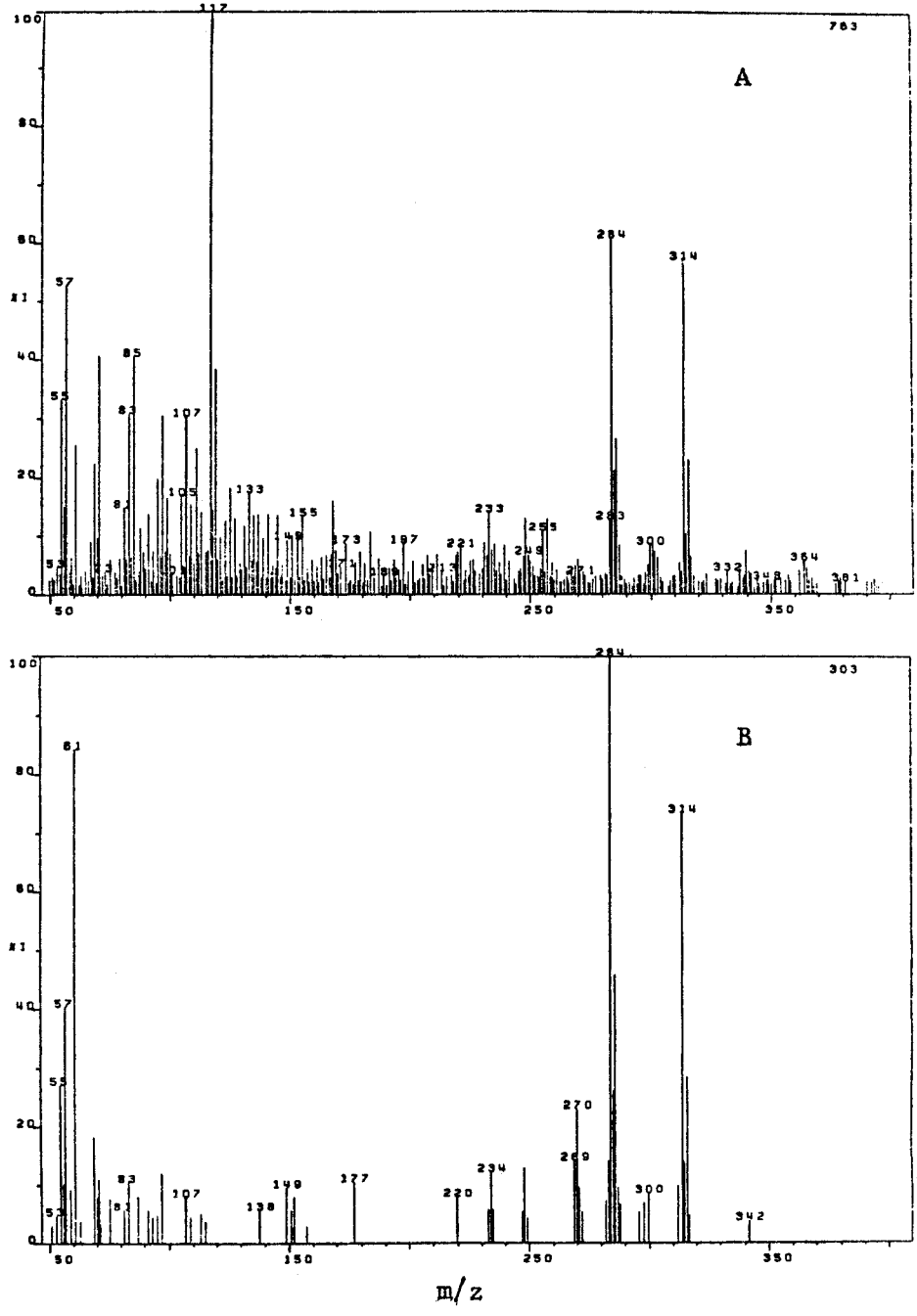
Figure 4. Chemical ionization mass spectra of the HPLC eluant extract corresponding to 3-hydroxyclonazepam and the known standard.

The HPLC eluant corresponding to 3-hydroxyclonazepam was collected, extracted, and direct probe analysis performed as described in Materials and Methods. The % intensity is plotted against the m/z ratio. The A spectrum is of the extract. The B spectrum is of authentic 3-hydroxyclonazepam standard from Roche.



hydroxyclonazepam standard. The mass spectrum shows major ions at 314 and 284. The ion at 314 is the m+1 ion of the thermal degradation product of 3-hydroxyclonazepam. The mechanism of the thermal degradation is by rearrangement to a six membered ring system followed by aromatization which results in a net loss of water. The 284 ion arises from the loss of an $H_2C=O$ side chain from the new ring to give the [(m+1)-30] ion (14). Under anaerobic conditions, 7-aminoclonazepam was the major metabolite (899.3 $\pm$ 86.6 pmoles/mg protein/min), along with a small amount of 3-hydroxyclonazepam (45.5 $\pm$ 14.2 pmoles/mg protein/min). The identity of the amine was also confirmed by mass spectrometry and was equivalent to published spectra (8).

As expected, no acetamido metabolites were observed in microsomal preparation, because acetyltransferase is a cytosolic enzyme. In vivo, one would expect to observe N-acetylated derivatives as well as other conjugated metabolites. Our results from rat liver microsomes contrast with those from the only published in vivo metabolic study, where only small amounts of the 3-hydroxyl derivatives of clonazepam were found.

In the future, we intend to use the described HPLC assay for the quantitation of metabolites in the urine and feces of germfree and conventional animals, in order to determine the relative contribution of the gut flora and mammalian enzynes to clonazepam nitro-reduction.

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