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# DETERMINATION OF CLONAZEPAM IN HUMAN PLASMA BY GAS CHROMATOGRAPHY–NEGATIVE ION CHEMICAL IONIZATION MASS SPECTROMETRY

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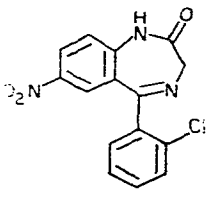
## SUMMARY

Our previously reported gas chromatographic–mass spectrometric assay for clonazepam using positive ion chemical ionization has been modified to employ negative ion chemical ionization. The sensitivity using this technique (< 0.1 ng/ml) is approximately twenty times better than the positive ion chemical ionization procedure with similar precision. The method was used to determine the clonazepam plasma concentration in one human for 96 h following a single 2-mg dose of clonazepam.

## INTRODUCTION

In a previous paper[1] describing a positive ion chemical ionization mass spectrometry (PICIMS) procedure for clonazepam (I), we suggested that negative ion chemical ionization mass spectrometry (NICIMS) might be even more useful for the determination of this antiepileptic agent. Hunt *et al.*[2] have reported NICIMS to be more sensitive for certain compounds than PICIMS. Recently Lewy and Markey[3] published a NICIMS assay for melatonin which can measure 1 pg of that compound in 1 ml of plasma. Based on its fine response when analyzed by electron-capture gas chromatography (GC)[4], clonazepam would be expected to readily undergo anion formation with the thermal electrons existing in a chemical ionization source. If observed, the increased sensitivity would be useful in measuring the single dose



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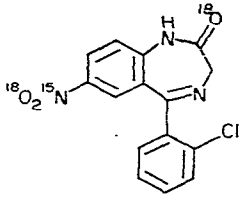

II

pharmacokinetic profile of clonazepam over more than one drug half-life. In addition, since clonazepam is often used in the treatment of epileptic infants and young children, a more sensitive assay would better cope with the small plasma samples available from these subjects.

This paper reports a GC–NICIMS assay for clonazepam in human plasma. For this assay a stable isotope analog of clonazepam, compound II, is used as the internal standard.

## EXPERIMENTAL

### *Instrumentation*

A Finnigan Model 3200 quadrupole chemical ionization mass spectrometer was modified to detect negative ions using the method of Stafford *et al.*[5]. The screen retainer on the top plate of the standard Finnigan continuous dynode electrode multiplier assembly was replaced by one of similar shape, but with a 0.25 in. × 0.36 in. opening. To the bottom of the screen retainer was spot welded a dynode ("conversion dynode") from a Finnigan Dumont "box and grid" electron multiplier. The standard Finnigan continuous dynode multiplier horn was replaced by a Model 4770 horn from Galileo Electro-Optics Corp. (Sturbridge, Mass., U.S.A.). The Model 4770 was suspended, with the opening of the horn facing the conversion dynode, by mounting the supplied retaining clip in existing holes in the ceramic support plates of the standard multiplier assembly. The "Faraday Cup" feed through was connected with bare copperwire to the multiplier top plate using one of the small nuts and bolts that hold the top plate to the ceramic support plates. The top of the horn was connected to the "minus high voltage" connection and the "signal out" lead was connected to the horn's electron collector. The voltage to the conversion dynode, *i.e.* top plate, was provided by connecting a Hewlett-Packard Model 6516A, 0–3000 V, power supply to the "Faraday Cup" feed through. To detect positive ions the conversion dynode is typically operated at −2000 V and the horn at −1700 V. To detect negative ions the conversion dynode is operated at +2000 V and the horn at −1700 V.

The ion energy, trap, repeller and lens leads were disconnected at the quadrupole flange. The trap and repeller feed thrus were connected to the ion energy feed thru which was itself connected to a ±30 V d.c. power supply Model 30-.6 (Hyperion, Watertown, Mass., U.S.A.). To stabilize this power supply a 1000 Ω, 5 W, resistor was placed across the output. This amplifier was typically operated at +10 V to detect positive ions and at −10 V to detect negative ions. The lens voltage was similarly provided by a Hewlett-Packard Model 6209B, ±320 V, d.c. power supply. Typical lens settings were +60 V in the negative ion mode and −60 V in the positive ion mode. The filament was operated at −100 V and an emission of 1000 mA. A 300 Gauss magnet was placed outside the vacuum on the quadrupole housing above the ion source. The magnet was moved until the position of optimum negative ion production was found.

Mass spectra were obtained using a Finnigan Model 6000 data system. Selected ion recordings were obtained using a Finnigan Promin® peak monitor. The GC conditions were as previously described[1]. Unlike the previous assay, ammonia was not used as reagent gas. Methane was used as both GC carrier gas and chemical ionization reagent gas.

*Methods and materials*

Methods and materials were identical to those reported in our PICIMS assay with the following exceptions. Compound II, purchased from KOR Isotopes (Cambridge, Mass., U.S.A.) was used as the internal standard. The compound was added to plasma to give a concentration of 10 ng/ml. No carrier substances were used and no attempt was made to analyze for the amino metabolite of clonazepam. Final residues were reconstituted in 100 $\mu$l of ethyl acetate prior to injection. For the assay, the ions at *m/e* 314 (clonazepam) and *m/e* 321 (compound II) were monitored in the GC effluent. The peak height ratios of *m/e* 314 and *m/e* 321 were determined and converted to compound amounts using standard curves. The standard curves were prepared from the observed ion ratios obtained from the analyses of control plasma spiked with 0, 0.5, 1, 2.5, 5, 10, 15, 20 and 25 ng of clonazepam. The best fit of the data was obtained by a linear least squares computer program. Once found, the slope and intercept values were used to generate a concentration from an unknown ion ratio.

Ten-ml blood samples were obtained from a 75-kg male at −0.5, 1, 3, 5, 7, 24, 48, 72 and 96 h after receiving a 2-mg oral dose of clonazepam powder with 200 ml of water. The blood was centrifuged and the plasma isolated and stored at −5°.

## RESULTS AND DISCUSSION

Initially an attempt was made to detect negative ions using a system similar to that reported by Smit *et al.*[6]. Our system had the added advantage of also using a floating coaxial feed thru to minimize the microphonic noise and charge leakage inherent in transferring small signals at high voltages with vacuum feed thrus. The system performed satisfactorily with concentrated samples. At the low sample levels associated with drug assays, the increased system noise, relative to positive ion operation, overwhelmed any sensitivity gain. This is consistent with the observation of Stafford *et al.*[5] that "a multiplier electrically biased for negative ions is thirty times noisier due to stray electrons than a normally biased or conventional positive ion electron multiplier".

Using the Stafford multiplier the noise in the positive and negative detection mode is similar when using the Promin to record the ions. This multiplier features a conversion dynode which converts negative ions to positive ions or positive ions to electrons. In the negative ion mode the generated positive ions are detected by the electron multiplier horn in the conventional manner. In the positive ion mode the generated electrons strike the horn and are multiplied. In this ion detection mode the conversion dynode acts like an additional "multiplication stage". We have operated this system for half a year. It has given good, if not better, performance in the positive ion mode than the standard multiplier system. Operation in the negative ion mode has been almost routine. The only problem observed has been a tendency for the multiplier to arc when the voltage difference between the conversion dynode and the multiplier goes above 4000 V. The arcing is observed as spikes on the selected ion chromatogram baselines. This problem hopefully can be solved by better mechanical construction or by finding a distance between the dynode and multiplier where the sensitivity is optimal without arcing.

We find negative ion sensitivity to be highly dependent on the cleanliness of the ion source and/or quadrupoles. With normal use, the instrument in our lab will give high sensitivity negative ion chemical ionization (NICI) results for approximately two weeks following source and quadrupole rod reconditioning. After this time the instrument can still be used for many more weeks of high sensitivity positive ion chemical ionization (PICI), but not NICI work. The reasons for this are unclear.

With methane NICI no ions attributable to methane could be observed in the mass spectra. The background ions are *m/e* 17 ($OH^-$), *m/e* 35 ($^{35}Cl^-$) and *m/e* 37 ($^{37}Cl^-$). Thus the often annoying intense reagent ion background found with PICI is absent with NICI. The ion source magnet has no effect on PICI operation but typically doubles negative ion sensitivity.

The NICI mass spectra of clonazepam and the clonazepam stable isotope analog used in the assay can be seen in Fig. 1. The spectra consist principally of an $M^-$ molecular

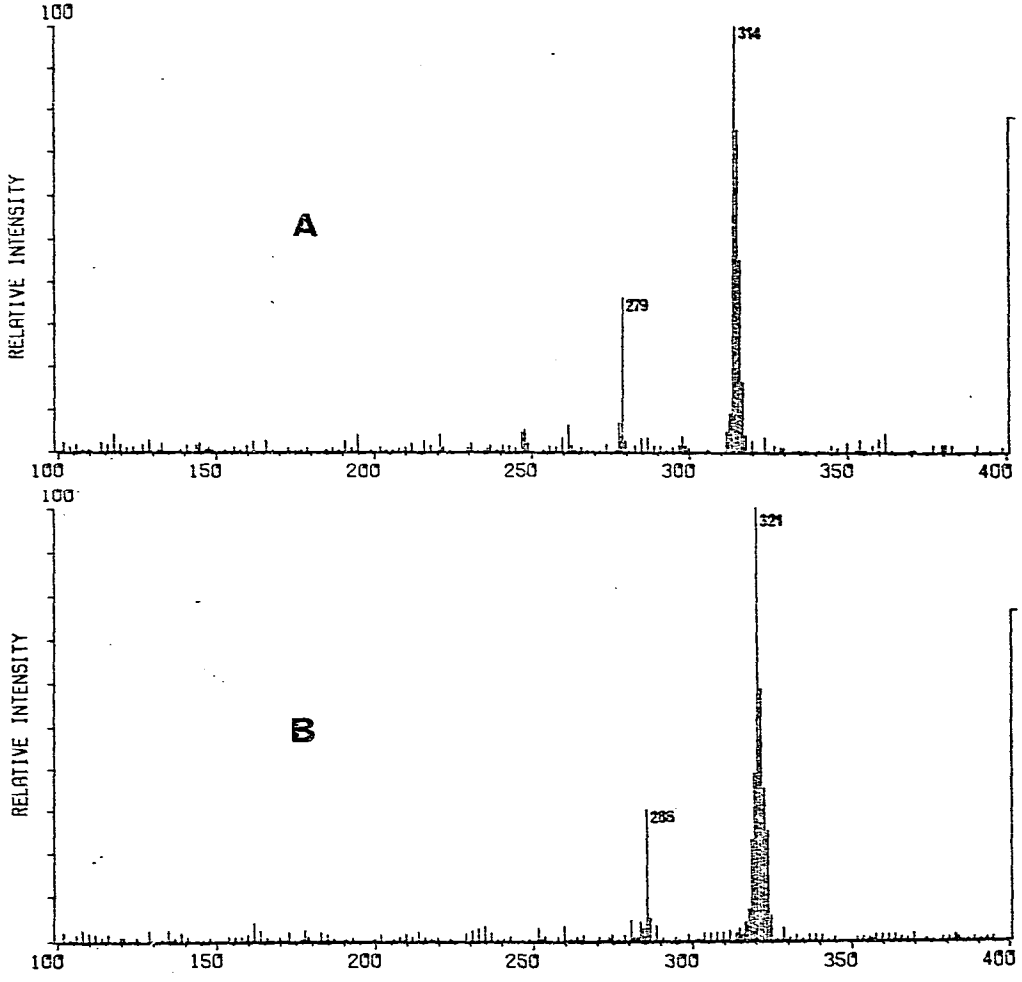


Fig. 1. Methane negative ion mass spectra of (A) clonazepam and (B) the internal standar compound II.

ion and $(M-H)^-$ and $(M-Cl)^-$ fragment ions. For the assay, the $(M-H)^-$ ions at *m/e* 314 and *m/e* 321 are monitored for clonazepam and its internal standard, respectively. The origin of the hydrogen lost to generate the $(M-H)^-$ ion is not known. Mass spectra of clonazepam specifically dideuterated on the carbon adjacent to the amide carbonyl suggest that the hydrogen lost does not come from that position. Whatever its origins, the intensity of the ion precludes the use of the single $^{15}N$ labelled internal standard we used in our PICI assay for clonazepam. The standard curve obtained using this internal standard in the NICI assay displayed severe curvative and a high zero concentration intercept value. Compound II as internal standard gave satisfactory standard curves. A typical standard curve using 10 ng of compound II is shown in Fig. 2. A least squares analysis of this curve gave a slope of 0.150 ± 0.002 and an intercept of 0.0063 ± 0.0155 ng. The correlation coefficient was 0.998.

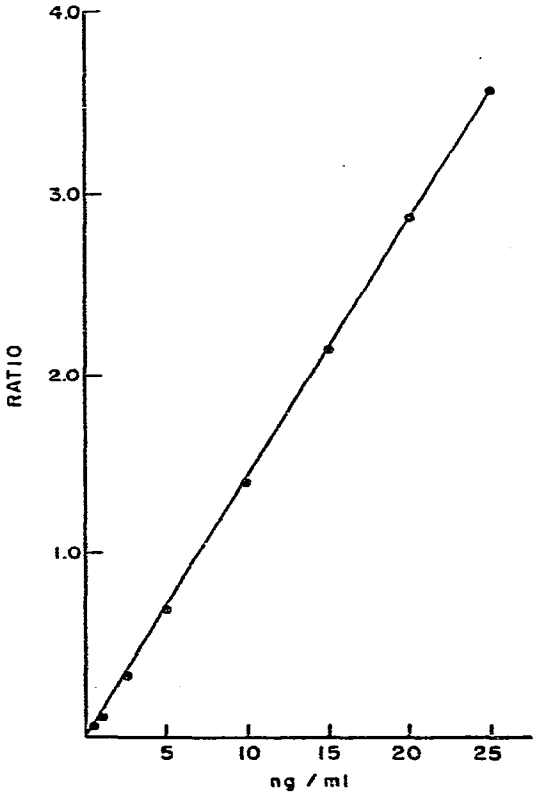


Fig. 2. A typical standard curve relating the ratio of *m/e* 314 to 321 with the addition of various amounts of clonazepam to control plasma. Internal standard, compound II, was present in a concentration of 10 ng/ml.

Ion chromatograms from the plasma analysis can be seen in Fig. 3. The dashed line is a tracing of the *m/e* 321 ion and the solid line is a tracing of the *m/e* 314 ion. All samples were spiked with 10 ng/ml of compound II. For the ion chromatogram from the analysis of the 0.5 ng/ml sample, the clonazepam peak (*m/e* 314) represents 0.02 ng of clonazepam injected. As can be seen, this peak shows virtually

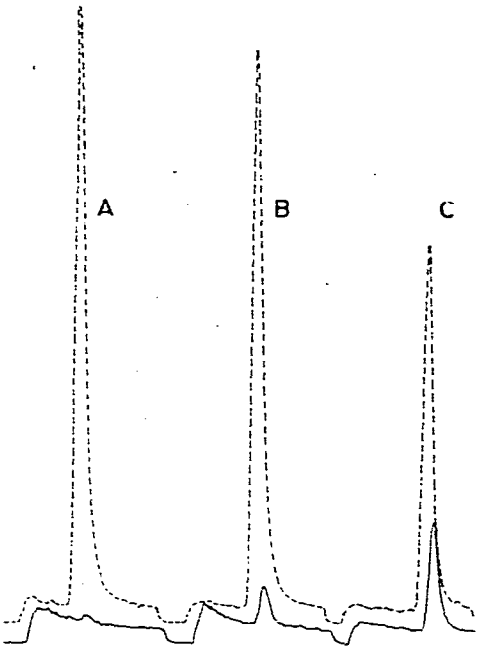


Fig. 3. Typical ion chromatograms from the analysis of plasma samples. – – –, $(M - H)^-$ ion of the internal standard (*m/e* 321). ——, $(M - H)^-$ ion of clonazepam (*m/e* 314). All samples were spiked with 10 ng/ml of internal standard and reconstituted in 100 μl of ethyl acetate. Retention time of clonazepam was 90 sec. (A) Control plasma spiked with 0 ng/ml of clonazepam, 5 μl were injected. (B) Plasma spiked with 0.5 ng/ml of clonazepam, 4 μl were injected. (C) Plasma sample from a patient who had received a 2-mg oral dose of clonazepam, 4 μl were injected. Clonazepam concentration in this sample was calculated to be 1.6 ng/ml.

no noise (signal-to-noise ratio > 100). Even at this sensitivity the ion chromatograms are remarkably simple. Indeed, to date one of the most impressive characteristics of NICI has been the lack of background ions even when using simple sample preparation procedures[7]. Based on the injection of many 1-ng standards over a period of several months clonazepam's $(M-H)^-$ ion response at *m/e* 315 is 15 to 25 times the PICI ion response at clonazepam's $MH^+$ ion at *m/e* 316. Assay sensitivity is limited by the amount of internal standard added. Using 5 ng/ml of compound II as internal standard the response at *m/e* 314 is typically 0.1 % or less of the response at *m/e* 321. Taking a value of twice the response of the blank as the sensitivity criteria the assay has an estimated sensitivity of 0.1 ng/ml.

In our PICI assay, a large amount of a carrier substance, structurally similar to clonazepam, was added to the biological samples before extraction to improve assay recoveries and chromatography. This compound did not interfere with the PICI ion chromatograms. When this was done in the NICI procedure, the carrier, which eluted considerably before clonazepam, caused a significant shift in the ion chromatogram baseline of clonazepam and the internal standard. Presumably the carrier depleted the ionizing thermal electrons from the source. For this reason the carrier substance was not used in the NICI assay.

To determine assay precision, six separate plasma samples were spiked to give

a concentration of 10 ng/ml of clonazepam and 5 ng/ml of compound II and were analyzed by the method described in this paper. The average concentration ± S.D. was determined to be 11.3 ± 0.8 ng/ml.

The results of the analysis of plasma samples from a subject who had received a single 2-mg dose of clonazepam can be seen in Fig. 4. The clonazepam concentration could be followed for 96 h. A clonazepam half-life of 22 h was obtained using the data from the 7–96 h post dosing samples. This clonazepam half-life falls in the range previously reported by Berlin and Dahlstrom[8] (19–60 h) and Kaplan *et al.*[9] (19–39 h).

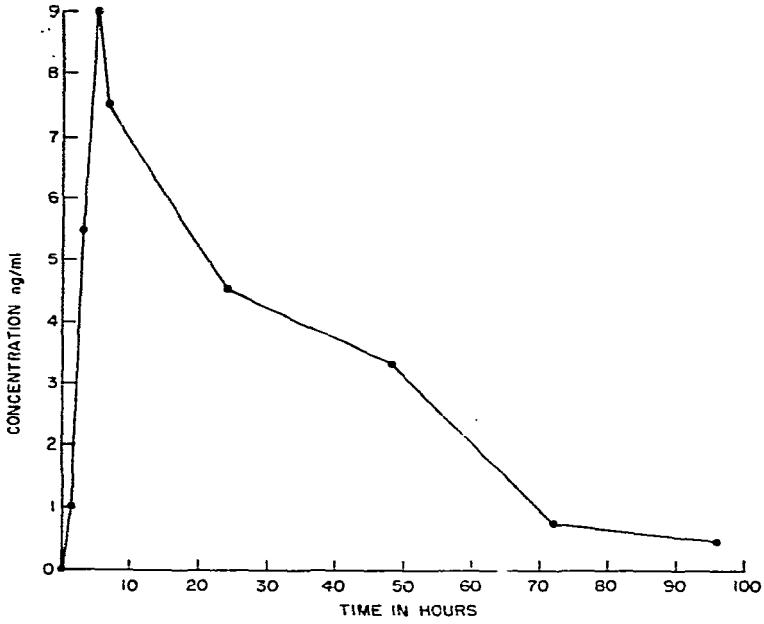


Fig. 4. Plasma concentration–time curve for a subject who had received a 2-mg oral dose of clonazepam.

Our experience with this assay over the last several months suggests that the NICI assay we describe here is the method of choice for the determination of clonazepam. The stable isotope analog insures assay accuracy and precision and NICI provides a high level of sensitivity and specificity. The specificity if provided not only by the chromatographic separation but also by the non-response of NICI to many of the materials present in biological media[7]. The sensitivity meets the criteria set out in our PICI assay article[1]. It allows the concentration of clonazepam to be determined over a period of several clonazepam half-lives following single dose administration and allows a decrease in the amount of plasma required for measuring steady state clonazepam plasma levels.

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