Quantitation of Clonazepam and Its Major Metabolite 7-Aminoclonazepam in Hair*

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Abstract

Clonazepam (CLO) is an anticonvulsant benzodiazepine approved by the Food and Drug Administration for use in the treatment of seizures. It produces pharmacological effects (depression, amnesia) similar to other compounds from the same therapeutic class, and in combination with alcohol, its CNS-depressant action can be significantly potentiated. As with some other benzodiazepines, CLO is a drug possibly used in “date-rape” situations. A method using solid-phase extraction followed by a highly sensitive negative chemical ionization gas chromatography-mass spectrometry for the simultaneous quantitation of CLO and its major metabolite 7-aminoceonazepam (7-ACLO) in hair was developed and validated. The method has potential application to alleged drug-facilitated rape cases. To determine the feasibility of detecting 7-ACLO and CLO in hair, specimens were collected from 10 psychiatric patients treated with CLO, divided into 2-cm segments, and analyzed. Standard curves for 7-ACLO (1–1000 pg/mg) and CLO (10–400 pg/mg) had correlation coefficients of 0.998. All precision and accuracy values were within acceptable limits. 7-ACLO was present in measurable quantities (1.37–1267 pg/mg) in 9 out of 10 patient samples. CLO concentrations in hair were much lower (10.7–180 pg/mg). In 4 out of 10 cases, CLO was not detected in hair. Two patients who had never been treated with CLO before received a single 2-mg dose of the drug. Approximately three weeks later, hair samples were collected, and measurable quantities of 7-ACLO (4.8 pg/mg) were detected in the first segment (proximal) of one of those samples, and traces of the drug were present in the other sample. We concluded that the 7-ACLO is being deposited in hair in much higher quantities than the parent drug and remains there for extended periods of time. Our study also indicates that it is possible to detect 7-ACLO after a single dose of CLO as in the typical date-rape scenarios.

Introduction

Clonazepam (Klonopin™, Clonex, Iktorivil, and Rivotril) (CLO) is a benzodiazepine that exhibits many of the characteristic pharmacologic properties of the class. It has been shown to decrease seizure activity, reduce anxiety, induce muscle weakness, and even induce sleep or hypnosis. It has been approved by the Food and Drug Administration for use in the treatment of seizures. However, physicians are also using CLO in the treatment of anxiety, mania, panic disorders, and schizophrenia because of its sedative and anxiolytic properties. Chemically, CLO is 5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one and has a molecular weight of 315 (1). It is available in tablets containing 0.5 mg, 1 mg, and 2 mg CLO. A single dose of CLO produces the desired effects in as little as 1–2 h, with the half life of the parent drug being 18–50 h. CLO is absorbed and distributed rapidly after oral administration with peak plasma levels obtained within 1–4 h. It is eliminated slowly, with an elimination half life of 30–40 h. CLO is highly metabolized, with less than 2% of the parent drug being excreted in the urine. The metabolism begins with the reduction of the 7-nitro group to the amine, and this amine is then acetylated to the acetamide. The compounds can also be hydroxylated at the C-3 position, which will result in their elimination as either the glucuronide or sulfate conjugate.

In the past few years, considerable information about so-called “date-rape drugs” and drug-facilitated sexual assault has accumulated, and in the same time an increase in the number of scientific publications on the subject has been observed (2–6). Because of its pharmacological effects, CLO has been identified as a compound potentially used in drug-facilitated rape (3,7,8) and has been classified as a Schedule IV substance. Very recently, Raymon et al. (8) reported involvement of flunitrazepam (among other benzodiazepines) in driving-under-the-influence cases in South Florida between 1995 and 1998 and a significant rise in CLO cases after 1998. Drug-facilitated sexual assault victims may not report the incident in a timely
manner because of fear, shame, and other psychological reasons. It is not unusual for a urine or blood analysis to provide a negative result because of the likelihood of a single, low-dose ingestion, urine dilution, and the length of time since the alleged incident. Analysis of hair using highly sensitive detection provides a solution to this problem. It is documented that 7-nitro benzodiazepines metabolize to 7-amino compounds and tend to incorporate into hair and remain there for much longer periods of time than either urine or in blood. Several forensically relevant benzodiazepines have been previously identified in plasma (9), urine (10), and hair (11–15) using negative ion chemical ionization gas chromatography–mass spectrometry (NCI-GC–MS). Ygges et al. (16) reported concentrations of several benzodiazepines in hair samples obtained from 21 corpses using electron ionization-GC–MS. Recently, LeBeau et al. (3) suggested that hair may be a valuable specimen in the cases of drug-facilitated sexual assault when reporting of the crime was delayed. Negruz et al. (17) described solid-phase extraction followed by highly sensitive NCI-GC–MS quantitation of flunitrazepam and 7-amino flunitrazepam in hair and applied it for the first time to the hair samples collected from alleged drug-facilitated rape victims.

The aim of study was to develop and validate a sensitive, precise, and accurate NCI-GC–MS method for the simultaneous detection of 7-aminoclonazepam (7-ACLO) and CLO in human hair with potential application to alleged drug-facilitated rape victims. To test the developed methodology, we analyzed hair samples collected from people treated with CLO. To our knowledge this is the first report on detection of CLO and its major metabolite 7-ACLO in human hair.

Experimental

Subjects and specimens

Ten subjects (4 women and 6 men, aged 26 to 66 years old) who were already being treated with CLO for various medical reasons in the Psychiatric Unit of the University of Illinois at Chicago Hospital participated in this study. All hair samples were collected between March and July 1999 using hair collection kits provided by the United States Drug Testing Laboratories, Inc. A single hair sample (equivalent to the thickness of a pencil) was collected close to the scalp from the back of each subject's head. The length of each hair sample was measured. The samples were stored at room temperature until analysis. Collection of a single hair sample from each subject was approved by the Institutional Review Board of the University of Illinois at Chicago.

Instrumentation

The GC–MS system consisted of a Hewlett-Packard 6890 series injector, an HP 6890 series GC system, and an HP 5973 mass selective detector with positive and negative ion chemical ionization capabilities (Hewlett Packard, Palo Alto, CA). An HP-5MS capillary column (30 m × 0.25 μm × 0.25 μm) was used for separation (Hewlett Packard, Palo Alto, CA). The hair pulverizer was acquired from Crescent (Lyons, IL), and the heating block was from Fisher Scientific (Itasca, IL). The Vac-Elut™ extraction manifold was from Analytical International (Varian, Harbor City, CA), and vacuum oven model 5831 (Napco®) was purchased from Fisher Scientific (Itasca, IL). The centrifuge model 5810 (Eppendorf-Netheler-Hinz GmbH, Germany) was acquired from Brinkmann Instruments (Westbury, NY), and the Meyer N-EVAP® analytical evaporator from Organamation Assoc. (Northborough, MA). The Aerograph water bath sonicator was purchased from Varian.

Chemicals and reagents

CLO (1 mg/mL in methanol), 7-ACLO (100 μg/mL in acetone), and the deuterated internal standard diazepam-d5 (100 μg/mL in methanol) were purchased from Radian International (Austin, TX). Methanol (high-performance liquid chromatography [HPLC] grade), hydrochloric acid (certified ACS Plus), glacial acetic acid (HPLC grade), methylene chloride (HPLC/GC–MS grade), isopropanol (HPLC grade), ethyl acetate (HPLC grade), and concentrated ammonium hydroxide (certified ACS Plus) were bought from Fisher Scientific. Hepafluorobutyril anhydride (HFBA) was purchased from Campbell Supply Co. (Rockton, IL). The HCX Isolute® 200-mg, 10-mL columns (International Sorbent Technologies) were purchased from Jones Chromatography (Lakewood, CO).

Standards and controls

The CLO (1 mg/mL in methanol) standard stock solution was diluted to 100 μg/mL, 10 μg/mL, 1 μg/mL, and 0.2 μg/mL. The 7-ACLO (100 μg/mL in acetone) standard stock solution was diluted to 10 μg/mL, 1 μg/mL, 0.1 μg/mL, and 10 ng/mL. The deuterated internal standard, diazepam-d5 (100 μg/mL in methanol), was diluted to 10 μg/mL and 1 μg/mL. All standards were diluted in their respective solvents.

An eight-point standard curve was made for 7-ACLO and a five-point curve for CLO by using 50-mg aliquots of the negative pulverized hair spiked with solutions of both drugs. The concentrations of the 7-ACLO in standard hair preparations were as follows: 1, 5, 10, 50, 100, 200, 500, and 1000 pg/mg of hair. The concentrations of CLO were 10, 50, 100, 200, and 400 pg/mg of hair. In addition, two levels of controls were prepared. The low controls for 7-ACLO (3 pg/mg) and CLO (30 pg/mg) were prepared by adding 15 μL of 10 pg/μL and 7.5 μL of 200 pg/μL, respectively, to 50-mg aliquots of negative hair. The high controls for 7-ACLO (800 pg/mg) and CLO (300 pg/mg) were prepared by adding 40 μL of 1000 pg/μL and 75 μL of 200 pg/μL, respectively, to 50-mg aliquots of negative hair.

Analytical procedure

The hair samples were divided into approximately 2-cm segments and pulverized, and 50-mg aliquots were analyzed. In one case, the hair sample was too short to be divided into segments. All other samples were divided into 2–8 segments. To each hair sample, standard, and control preparations, 30 μL of 1-μg/mL of diazepam-d5 (600 pg/mg) was added. Methanol (3 mL) was added to the hair, and it was sonicated for 1 h. The samples were centrifuged for 5 min at 400 × g, and the methanol layer was decanted and transferred to clean test tubes for storage in the refrigerator. To the remaining hair, 0.1N hydrochloric acid (3 mL) was added, and the specimens were incubated overnight at 55°C after being sealed and vortex mixed.
The test tubes were removed from the heating block and centrifuged for 5 min at 400 x g. The methanol and acid were combined, and 1.93M glacial acetic acid (1 mL) and deionized water (9 mL) were added. Mixed-mode Isolute HCX solid-phase extraction columns were conditioned with the following, never allowing them to dry: methanol (3 mL), deionized water (3 mL), and 1.93M glacial acetic acid (1 mL). The sample was then added to the column and drawn through slowly. The column was allowed to dry for approximately 2 min. The bed of the column was washed with 3 mL of deionized water (dried for 1–2 min), 1 mL of 0.1N hydrochloric acid (dried for 1–2 min), and 3 mL of methanol (dried for 5 min). The collection tubes were placed in the rack, and the drugs were eluted using a mixture of methylene chloride/isopropanol/ammonium hydroxide (78:20:2, v/v/v) (3 mL). The eluent was then evaporated to dryness using a stream of nitrogen.

The dry residue was reconstituted in 50 μL of ethyl acetate and transferred to autosampler vials. The extract was evaporated to dryness in the vacuum oven at 60°C. The samples were derivatized using 50 μL of HFBA at 60°C for 30 min in the sealed vials. After incubation, the derivatizing agent was evaporated in the vacuum oven at 60°C, and the dry residue was reconstituted in 25 μL of ethyl acetate.

Chromatographic method

The injector was operated in the splitless mode at 240°C, and the injection volume was 1 μL. Ultra-high purity helium (99.999%) was used as the carrier gas at a flow rate of 1.2 mL/min. The initial GC oven temperature of 60°C was held for 1 min, and then increased at a rate of 30°C/min until the final temperature of 310°C was attained. The final temperature was held for 3 min. The total run time for one injection was 12.33 min. Methane (ultra high purity, 99.999%) was used as reagent gas at an apparent pressure of 3.7 x 10⁻⁴ Torr in the ion source (methane flow 3.25 mL/min.) The MS ion source temperature was 250°C, and the quadrupole temperature was 106°C. The electron multiplier voltage was set at +400V above the NCI-tune voltage.

The mass selective chemical ionization detector was monitoring negative ions (NCI) in the selected ion monitoring (SIM) mode. The solvent delay was 9 min. The detector was turned off after 11.1 min. The following ions were monitored and used for quantitation: m/z 315 and 279 for CLO, m/z 461 and 445 for 7-ACLO, and m/z 289 for diazepam-d₅. The dwell time for all ions was 20 ms. The single-taper deactivated liners with glass wool were used. It was necessary to change the liner daily because CLO is particularly sensitive to active sites.

Precision and accuracy

Quantitation of CLO and 7-ACLO was performed by the internal standard method. An eight-point standard curve for 7-ACLO and five-point standard curve for CLO were prepared by linear least-square regression analysis of the ratio of the peak area of 7-ACLO and CLO to the peak area of the internal standard, diazepam-d₅. Peak-area ratios were determined for the control hair preparations. Control concentrations were calculated from the standard curve values.

Intraday variability was ascertained by analyzing three replicates of low controls (30 pg/mg, 3 pg/mg) and four high controls (300 pg/mg, 800 pg/mg) for CLO and 7-ACLO, respectively. Interday variability was ascertained over a period of eight weeks. The mean measured concentrations and standard deviations

Figure 1. Selected ion chromatograms of the blank hair (A, m/z 461; B, m/z 289), and blank hair spiked with 7-ACLO at a concentration of 3 pg/mg (C, m/z 461; D, m/z 289) and CLO.
were calculated based on the inter- and intraday variability populations.

The percent relative accuracy was calculated by the following equation: 

\[
\frac{(\text{Mean Measured Concentration} - \text{Theoretical Concentration})}{\text{Theoretical Concentration}} \times 100\%
\]

All data were acquired and analyzed by HP software, Enhanced G1701BA ChemStation (ver B.00.00 for Windows NT ver 4.0).

Results

The retention times of 7-ACLO, CLO, and diazepam-d₅ were approximately 10.10 min, 10.77 min, and 9.37 min, respectively. All chromatograms were recorded over the time range of 9.00 to 11.10 min. Figure 1 shows selected ion chromatograms (m/z 461, m/z 289) of the extract from blank hair (Figure 1A and B), and blank hair spiked with 7-ACLO and diazepam-d₅ (Figure 1C and D). The concentration of 7-ACLO was 3 pg/mg. Figure 2 shows the selected ion chromatograms (m/z 461) of the extract from the proximal hair segment (Figure 2A) and distal segment #8 (Figure 2B) collected from subject #9. The concentration of 7-ACLO in segment #1 was 1267 pg/mg and in segment #8, 70 pg/mg. CLO was detected in the first three segments of the hair sample collected from subject #9 (180, 32, and 27 pg/mg, respectively). The selected ion chromatogram (m/z 461) of the extract from hair sample (proximal segment) from subject #2 is presented in Figure 3. This patient received a single 2-mg dose of CLO approximately three weeks earlier. The concentration of 7-ACLO in the proximal hair segment was 4.8 pg/mg. The distal hair segment was negative for

\[\text{Figure 2. Selected ion chromatograms (m/z 461) of the extract from the proximal hair segment, 7-ACLO concentration 1267 pg/mg (A), and distal segment, 7-ACLO concentration 70 pg/mg (B), collected from subject #9.}\]

\[\text{Figure 3. Selected ion chromatogram (m/z 461) of the extract from proximal hair segment collected from subject #2 (7-ACLO concentration 4.8 pg/mg).}\]

\[\text{Figure 4. Standard curves for CLO (A) and 7-ACLO (B).}\]
7-ACLO. CLO was not detected in either of these segments. Figure 4 presents the standard curves for CLO (Figure 4A) and 7-ACLO (Figure 4B). They were linear over the range of drugs assayed (1 pg/mg to 1000 pg/mg for 7-ACLO and 10 pg/mg to 400 pg/mg for CLO) and had correlation coefficients of 0.998. The limits of quantitation were 1 pg/mg for 7-ACLO and 10 pg/mg for CLO for 50-mg samples. Both limits of quantitation were arbitrarily established to be the lowest concentrations on the corresponding standard curves. The respective limits of detection for CLO and 7-ACLO were 5 pg/mg and 0.4 pg/mlg, which were the lowest concentrations of drug at which the signal-to-noise ratio was 3:1.

Tables I and II present the accuracy and precision of the CLO and 7-ACLO control hair preparations, respectively. The intraday variability was determined by analyzing three low and four high replicates of controls (30 and 300 pg/mg of hair for CLO and 3 and 800 pg/mg of hair for 7-ACLO) prepared in hair on a single day. The interday variability was determined over a period of 8 weeks on 10 separate days.

Figure 5 presents concentrations of 7-ACLO in hair samples collected from subjects #1 and #2. Both subjects received a single 2-mg dose of CLO approximately three weeks before hair collection. For subject #1 only traces (below quantitation limit) of 7-ACLO were detected; for subject #2 4.8 pg/mg of 7-ACLO were detected. The concentrations of 7-ACLO in the remaining eight hair samples are presented in Figure 6. 7-ACLO was present in measurable quantities in 9 out of 10 hair samples collected, and the concentration range was 1.37–1267 pg/mg. Figure 7 shows concentrations of parent drug, CLO, in study samples. It was not detected in 4 out of 10 samples, and the concentration range was 10.7–180.6 pg/mg.

### Table I. Accuracy and Precision of Clonazepam Hair Preparations (pg/mg)

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<th>Parameter</th>
<th>Low control</th>
<th>High control</th>
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<td>Theoretical concentration</td>
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<td>300</td>
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<td>Intraday variability</td>
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<tr>
<td>Mean measured concentration</td>
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<td>% CV</td>
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<tr>
<td>% Relative accuracy</td>
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<td>-3.73</td>
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<td>Interday variability</td>
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<tr>
<td>Mean measured concentration</td>
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<tr>
<td>% CV</td>
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<td>% Relative accuracy</td>
<td>6.27</td>
<td>-2.84</td>
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### Table II. Accuracy and Precision of 7-Aminoclonazepam Hair Preparations (pg/mg)

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<td>Theoretical concentration</td>
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<td>Mean measured concentration</td>
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<td>% CV</td>
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<td>% CV</td>
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<td>% Relative accuracy</td>
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<td>4.59</td>
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**Discussion**

Clonazepam, together with flunitrazepam and nitrazepam, belongs to the group of 7-nitro-1,4-benzodiazepin-2-ones (1). In the human body they all undergo complex biotransformation, which includes reduction of the nitro group to the amine (1,13). All three benzodiazepines and their 7-amino metabolites are...
basic; therefore, they accumulate in hair and remain present for extended periods of time. The presence of flunitrazepam and 7-aminoflunitrazepam in hair in relatively high quantities has been well documented (12–17). Cirimele et al. (13) found 26 hair specimens collected from corpses positive for 7-aminoflunitrazepam, flunitrazepam, or both. Flunitrazepam only was found in 14 samples. All the specimens were positive for 7-aminoflunitrazepam. Cirimele et al. (14) also described the determination of chronic flunitrazepam abuse by analysis of a 10-cm hair strand collected from a chronic drug user and divided into 3-cm segments. In another study (17), concentrations of 7-aminoflunitrazepam in two postmortem hair samples were found to be higher than corresponding flunitrazepam levels. NCI-GC-MS offers much better sensitivity than the conventional EI-GC–MS and has been successfully applied to the detection of various benzodiazepines in human hair (12–15,17). CLO and 7-ACLO, however, have not been included in those studies. To our knowledge, this is the first report on simultaneous detection and sensitive quantitation of CLO and its major metabolite 7-ACLO in segmented human hair.

The number of papers on detection of CLO and its metabolites, including 7-ACLO, in biological specimens is very limited. De Silva et al. (1) described serious difficulties with GLC analysis of blood and urine samples for CLO. Song et al. (9) reported several analytical problems with the quantitative determination of CLO in plasma using NCI-GC–MS, such as binding of the compound to the analytical column, injection port, ion source and quadrupole. In their study, CLO was found to be active even as a TMS derivative. In our study, the limit of quantitation for CLO was 10 pg/mg, and it was necessary to change the liner daily (practically every 15 injections) in order to keep method validation parameters within acceptable limits because CLO is particularly sensitive to active sites. The standard curve for CLO was linear over the concentration range studied. The standard curve for 7-ACLO had a wide linear concentration range (1 pg/mg–1000 pg/mg). Both standard curves had a correlation coefficient of 0.998. The limit of quantitation for CLO established in this study was considerably higher than previously described for flunitrazepam in hair using similar analytical methodology (17). In addition, our study revealed that the concentrations of 7-ACLO in hair are much higher than CLO. In subjects #9 and #10, 7-ACLO was found in the distal segments that were over 20 cm in length (Figure 5); the concentrations of 7-ACLO were 70 pg/mg.

Conclusions

The analytical method presented in this paper for the determination of CLO and its major metabolite 7-ACLO in hair employs solid-phase extraction and GC–NICI-MS, is extremely sensitive, and allows accurate and precise quantitation of both drugs in hair samples collected from CLO users. The 7-ACLO concentrations in hair were higher than concentrations of the parent drug, and 7-ACLO remains in hair for extended periods of time. The developed methodology can possibly be applied to hair samples collected after administration of a single dose of the drug as in drug-facilitated sexual assault scenarios.

Acknowledgments

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References


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