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DETECTION OF "DATE-RAPE" DRUGS IN HAIR AND URINE

Final Report

Report Prepared for:	National Institute of Justice Office of Justice Programs U.S. Department of Justice 810 7th Street, N.W. Washington, D.C. 20531
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Award Number:	98-LB-VX-K020
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OBJECTIVES OF THE PROJECT

The objective of this project was to develop a methodology to detect flunitrazepam and its major metabolite 7-aminoflunitrazepam in the hair of victims of drug-facilitated sexual assault. The extremely sensitive confirmatory assay was expanded to include other benzodiazepines such as diazepam, clonazepam, 7-aminoclonazepam, triazolam and alprazolam but also gamma hydroxybutyrate (GHB), ketamine and scopolamine. In addition, a controlled clinical study was performed to test how long after administration of a single dose of Rohypnol®, flunitrazepam and its major metabolite 7-aminoflunitrazepam can be detected in urine and hair of participating volunteers using previously developed extremely sensitive NCI-GC-MS confirmatory technique and a commercially available, very sensitive micro-plate EIA.

I Detection of Flunitrazepam and 7-Aminoflunitrazepam in Urine and Hair After a Single Dose of Rohypnol®

1. <u>Detection of Flunitrazepam and 7-Aminoflunitrazepam in Urine</u>

Subjects and Specimens

Ten volunteers (8 women and 2 men, age 22 to 48 years old) participated in this study. They were admitted to the Psychiatric Unit of the University of Illinois at Chicago Hospital between June 1 and June 30, 1999, and a single 2 mg oral dose of Rohypnol® (Flunitrazepam) was given to each subject at approximately 8:30 AM. The subjects were housed in the Psychiatric Unit of the UIC Hospital for approximately 8 hours following drug administration. As we previously reported, few hair samples collected from the fist group of volunteers were inadvertently misplaced in UIC hospital. The study was repeated with another group of ten volunteers (8 women and 2 men, age 21 to 49 years old), admitted to the UIC Hospital Psychiatric Unit between July and August, 1999. Urine samples were collected according to the following schedule: one sample prior to drug administration, and 6 hours, 1 day, 3, 5, 8, 10, 14, 21 and 28 days after Rohypnol® intake. All samples were stored frozen until analyzed. This experiment was reviewed and approved by the Food and Drug Administration (IND number 57,284) and by the Institutional Review Board of the University of Illinois at Chicago (IRB number H-97-996).

Micro-Plate Enzyme Immunoassay

Materials

Mixed-mode solid-phase extraction columns (200 mg;10 mL; Isolute® HCX) were obtained from Jones Chromatography, Lakewood, CO. The 7-aminoflunitrazepam standard was obtained from the Radian Corporation, Austin, TX. STC Technologies, Inc., Bethlehem, PA generously loaned the micro-plate washer and reader, supplied Benzodiazepine Urine Micro-Plate kits and

Stabilzyme buffer. Glacial acetic acid, sodium acetate, methanol, hydrochloric acid, methylene chloride, isopropanol and concentrated ammonium hydroxide were purchased from Fisher Scientific, Itasca, IL, and they were of HPLC grade or better. β-glucuronidase (Type H-2 crude solution, 110,350 units/mL from *Helix pomatia*) was purchased from Sigma (St. Louis, MI).

Screening of Urine for Benzodiazepines

In order to optimize the micro-plate enzyme immunoassay for 7-aminoflunitrazepam and flunitrazepam and to assure the longest possible time of detection of both compounds in urine after a single dose of Rohypnol®, all urine samples collected from one volunteer were analyzed using three separate preparation steps:

1) No pre-treatment: Urine (25 µL) was used as the specimen with no other pre-treatment.

2) Hydrolysis: β-glucuronidase (100 µL) was added to urine (2 mL) and 0.1 M sodium acetate buffer (1 mL; pH 4.5). The sample was sonicated and incubated for 2 hours at 37^0C. The sample was cerntrifuged (2500 rpm; 5 min). An aliquot (25 µL) of the supernatant was used as the specimen.

3) *Hydrolysis and solid-phase extraction*: β-glucuronidase (100 µL) was added to urine (2 mL) and 0.1 M sodium acetate buffer (2 mL; pH 4.5). The sample was sonicated and incubated for 2 hours at 37^0C. Following incubation, mixed-mode solid-phase extraction columns were conditioned with methanol (3 mL), deionized water (3 mL) and 1.93 M acetic acid (1 mL). The sample was added to the column through a filter, and drawn through the sorbent bed slowly. The bed was washed with deionized water (3 mL), 0.1 N hydrochloric acid (1 mL) and methanol (3 mL). Collection tubes were placed into the rack and the drugs were eluted in methylene chloride:isopropanol:ammonium hydroxide (78:20:2, v/v/v; 3 mL). The eluent was evaporated to dryness at 60^0C and the residue reconstituted in 50% Stabilzyme buffer (300 µL).

The specimen, calibrator or control (25 µL) was added to each well of the micro-plate. The enzyme conjugate (100 µL) was added and the plate was incubated at room temperature in the dark for 30 minutes. The plate was emptied and washed with deionized water (6 x 300 µL). Substrate reagent was added (100 µL). The plate was again incubated in the dark, at room temperature, for 30 minutes. After incubation, stopping reagent was added (100 µL) and the absorbance was read at 450 nm using a micro-plate reader. Calibrators supplied with the Benzodiazepine Urine Assay at concentrations of 0, 100, 300 and 1000 ng/mL oxazepam equivalents were run with each plate along with negative and spiked positive controls. Spiked positive controls were prepared by adding 500 ng of 7-aminoflunitrazepam to drug free urine (2 mL) giving a concentration of 250 ng/mL.

Chemical Ionization Gas Chromatography-Mass Spectrometry

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 Company, Wilmington, DE). An HP-5MS capillary column (30 m x 250 µm x 0.25 µm) was used for separation (Hewlett Packard Company, Wilmington, DE). The heating block was from Fisher Scientific (Itasca, IL) and vacuum oven model 5831 (Napco®) purchased from Fisher Scientific (Itasca, IL). The shaker bath model 50 was from Precision Scientific Company, Chicago, IL. The Vac-Elut™ extraction manifold was from Analytical International (Varian, Harbor City, CA), the centrifuge model 5810 (Eppendorf-Netheler-Hinz GmbH, Germany) was acquired from Brinkmann Instruments, Inc., Westbury, NY, and the Meyer N-EVAP® analytical evaporator was from Organomation Assoc., Inc. (Northborough, MA).

Materials and Reagents

Flunitrazepam (1 mg/mL in methanol), 7-aminoflunitrazepam (100 μg/mL in acetonitrile), and the deuterated internal standards D_7 flunitrazepam (100 μg/mL in methanol) and D_7 7-aminoflunitrazepam (100 μg/mL in acetonitrile) were all purchased from Radian International (Austin, TX). Methanol (HPLC grade), hydrochloric acid (certified A.C.S. Plus), glacial acetic acid (HPLC grade), methylene chloride (HPLC/GC/MS grade), isopropanol (HPLC grade), ethyl acetate (HPLC grade), concentrated ammonium hydroxide and sodium acetate (certified A.C.S. Plus) were bought from Fisher Scientific (Itasca, IL). Heptafluorobutyric anhydride (HFBA) was purchased from Campbell Supply Company (Rockton, IL). β-glucuronidase (Type H-2 crude solution, 110,350 units/mL from *Helix pomatia*) was purchased from Sigma (St. Louis, MI). The HCX Isolute® 10 mL 200 mg columns (International Sorbent Technologies) were purchased from Jones Chromatography (Lakewood, CO).

Standards and Controls

The flunitrazepam (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-aminoflunitrazepam (100 μg/mL in acetonitrile) standard stock solution was diluted to 10 μg/mL, 0.1 μg/mL, and 10 ng/mL. The D_7 flunitrazepam (100 μg/mL in methanol) and D_7 7-aminoflunitrazepam (100 μg/mL in acetonitrile) deuterated internal standards were diluted to 10 μg/mL and 100 ng/mL. All standards were diluted in their respective solvents.

A seven point standard curves were made for both flunitrazepam and 7-aminoflunitrazepam using negative urine spiked with solutions of both drugs. The concentrations of the flunitrazepam in standard urine preparations were as follows: 100, 300, 500, 700, 1000, 1500, and 2000 pg/mL of

urine. The concentrations of 7-aminoflunitrazepam were 10, 50, 100, 150, 200, 500, and 1000 pg/mL of urine. In addition, two levels of controls were prepared. The low controls for flunitrazepam (200 pg/mL) and 7-aminoflunitrazepam (30 pg/mL) were prepared by adding 40 μL of 10 pg/μL and 6 μL of 10 pg/μL, respectively, to 2 mL aliquots of negative urine. The high controls for flunitrazepam (1,800 pg/mL) and 7-aminoflunitrazepam (800 pg/mL) were prepared by adding 18 μL of 200 pg/μL and 16 μL of 100 pg/μL, respectively.

Analytical Procedure

The urine samples were thawed and 2 mL aliquots were analyzed. To the urine samples, standard and control preparations, 15 μL of 100 pg/μL of D_7 flunitrazepam and 5 μL of 100 pg/μL of D_7 7-aminoflunitrazepam were added. To all urine samples, 0.1 N acetate buffer (pH 4.5, 1 mL) and 100 μL of β-glucuronidase were added and the specimens were incubated for 1.5 hour at 37^0C, after being sealed and vortexed. The test tubes were removed from the shaker bath and 1.93 M glacial acetic acid (1 mL) and deionized water (9 mL) were added. All samples were extracted using mixed-mode Isolute® HCX solid-phase extraction columns as described for micro-plate enzyme immunoassay screening. The eluent was evaporated to dryness at room temperature 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 60^0C. The samples were derivatized using 50 μL of HFBA at 60^0C for 30 minutes in the sealed vials. After incubation, the derivatizing agent was evaporated in the vacuum oven (60^0C) and the dry residue reconstituted in 25 μL of ethyl acetate.

Chromatographic Method

The injector was operated in the splitless mode and the injection volume was 1 µL. The injector temperature was 240^0C. Ultra high purity helium (99.999%) was used as the carrier gas at a constant flow of 1.2 mL per minute. The initial GC oven temperature of 60^0C was held for one minute, and then increased at a rate of 30^0C per minute until the final temperature of 310^0C was attained. The final temperature was held for three minutes. The total run time for one injection was 12.33 minutes. The transfer line temperature was maintained at 280^0C. Methane (ultra high purity - 99.999%) was used as reagent gas at an apparent pressure of 3.8×10^{-4} Torr in the ion source (methane flow 3.25 mL/min.) The MS ion source temperature was 250^0C and the quadrupole temperature was 106^0C. The electron multiplier voltage was set at +400V above the NCI-tune voltage.

The mass selective chemical ionization detector was monitoring negative ions (NCI) and it was operating in the selected ion monitoring (SIM) mode. The solvent delay was nine minutes. The following ions were monitored and used for quantitation: for flunitrazepam *m/z* 313 and 297, D_7 flunitrazepam *m/z* 320, for 7-aminoflunitrazepam *m/z* 459 and 441, and for D_7 7-aminoflunitrazepam *m/z* 466. The dwell time for the ions *m/z* 313, 297, 459 and 441 was 20 ms, and for *m/z* 320 and 466 was 50 ms. It was necessary to change the liner frequently since flunitrazepam is particularly sensitive to active sites.

Precision and Accuracy

Quantitation of flunitrazepam and 7-aminoflunitrazepam was performed by the internal standard method. A seven-point standard curve was prepared by linear least square regression analysis of the ratio of the peak area of flunitrazepam to the peak area of the internal standard, D_7

flunitrazepam. A separate seven-point standard curve was also prepared for 7-aminoflunitrazepam with D_7 7-aminoflunitrazepam as the internal standard. Peak area ratios were determined for the controls. Control concentrations were calculated from the standard curve values.

Intra-day variability was ascertained by analyzing three replicates of low controls (200 pg/mL, 30 pg/mL) and four high controls (1,800 pg/mL, 800 pg/mL) for flunitrazepam and 7-aminoflunitrazepam, respectively. Inter-day variability was ascertained over a period of six weeks. The mean measured concentrations and standard deviations were calculated based on the inter- and intra-day variability populations. The percent relative accuracy was calculated by the following equation: [(Mean Measured Concentration - Theoretical Concentration) / Theoretical Concentration] x 100%. All data were acquired and analyzed by HP software, Enhanced G1701BA ChemStation version B.00.00 for Windows NT ver 4.0.

Results

Micro-Plate Enzyme Immunoassay

We suggest this commercially available, highly sensitive assay for use in sexual assault investigations if the use of flunitrazepam is suspected, even though the benzodiazepine micro-plate enzyme immunoassay kit itself is targeted towards oxazepam. The cross-reactivity of structurally related compounds, including 7-aminoflunitrazepam, flunitrazepam main urinary metabolite, is 156% oxazepam equivalents (package insert information). Figure 1 presents the results of analysis of urine samples collected from the same subject and analyzed by commercially available micro-plate enzyme immunoassay using three different preparation steps: no pre-treatment, enzymatic hydrolysis and hydrolysis followed by solid-phase extraction. For all three sample preparation procedures the highest concentration of benzodiazepines (the lowest absorbance value) was observed

24 hours after administration of a single dose of Rohypnol®. The absorbance values, which ranged from1.25 to1.79 (area between two lines) represent urine samples negative for flunitrazepam and its metabolites. Figure 1 clearly shows, that the extraction step significantly increases the concentration of analytes and expands the detection time to 8-10 days. Definitive detection of flunitrazepam-related compounds in unextracted urine and urine hydrolyzed without extraction was possible only during the first 5 days after drug administration

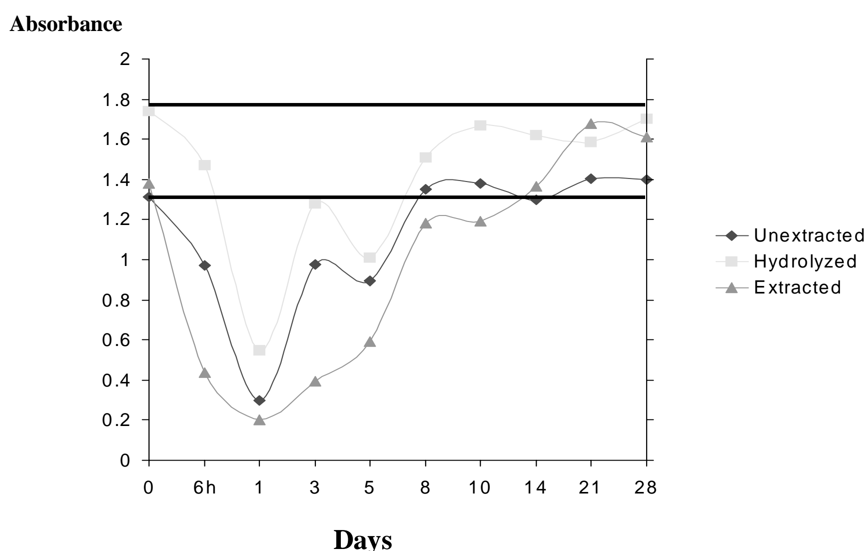

Figure 1. Micro-plate enzyme immunoassay analysis of urine samples collected from the same subject using three different sample preparation steps: no pre-treatment, enzymatic hydrolysis, enzymatic hydrolysis followed by solid-phase extraction.

Chemical Ionization Gas Chromatography-Mass Spectrometry

Tables 1 and 2 present the accuracy and precision of the flunitrazepam and 7-aminoflunitrazepam control urine preparations, respectively. Intra-day variability was determined

by analyzing three replicates of low controls (200 pg/mL, 30 pg/mL) and four high controls (1,800 pg/mL, 800 pg/mL) for flunitrazepam and 7-aminoflunitrazepam, respectively. Inter-day variability was ascertained over a period of six weeks.

Table 1. Accuracy and Precision of Flunitrazepam Urine Preparations (pg/mL)

Parameter	Low Control	High Control
Target Concentration	200	1,800
INTRA-DAY	N=3	N=4
Mean Measured Conc. (±S.D.)	166.56 (5.97)	1,883.61 (127.31)
% Coefficient of Variation	3.58	6.76
% Relative Accuracy	-16.72	4.64
INTER-DAY	N=20	N=16
Mean Measured Conc. (±S.D.)	213.12 (30.98)	1,740.76 (280.32)
% Coefficient of Variation	14.54	16.10
% Relative Accuracy	6.56	-3.29

Table 2. Accuracy and Precision of 7-aminoflunitrazepam Urine Preparations (pg/mL)

Parameter	Low Control	High Control
Target Concentration	30	800
INTRA-DAY	N=3	N=4
Mean Measured Conc.		
(±S.D.)	27.87 (4.89)	816.41 (60.81)
% Coefficient of Variation	17.55	7.45
% Relative Accuracy	-7.10	2.05
INTER-DAY	N=17	N=20
Mean Measured Conc.		
(±S.D.)	36.30 (5.88)	773.68 (82.72)
% Coefficient of Variation	16.20	10.69
% Relative Accuracy	21.00	-3.29

The retention times of 7-aminoflunitrazepam, D_7 7-aminoflunitrazepam, flunitrazepam and D_7 flunitrazepam were approximately 9.53, 9.52, 9.94 and 9.92, minutes, respectively. All chromatograms were recorded over the time range 9.00 to 11.00 minutes. Standard curves for flunitrazepam and 7-aminoflunitrazepam were linear over the range of drugs assayed (10 pg/mL to 1000 pg/mL for 7-aminoflunitrazepam and 100 pg/mL to 2000 pg/mL for flunitrazepam) and had correlation coefficients 0.977 and 1.000, respectively. The limit of quantitation for 7-aminoflunitrazepam was 10 pg/mL and for flunitrazepam 100 pg/mL for a 2 mL sample. Both limits

of quantitation were arbitrarily established to be the lowest concentrations on the corresponding standard curves. The limit of detection for flunitrazepam was 3 pg/mL and for 7-aminoflunitrazepam 50 pg/mL, which was the lowest concentrations of drugs at which the signal-to-noise ratio was 3:1.

In three subjects (volunteers #2, #4 and #8), 7-aminoflunitrazepam was detected throughout the entire 28-day study period (detected concentrations were 16.5 to 22.1 pg/mL). Figure 2 presents the elimination of 7-aminoflunitrazepam in urine in all subjects. Flunitrazepam was detected in urine collected from seven volunteers (100 pg/mL to 458 pg/mL) up to three days and in one subject five days after Rohypnol® administration (Figure 3). Urine samples collected from two volunteers were positive for flunitrazepam only during the first 24 hours. Flunitrazepam concentrations in urine never exceeded 3 ng/mL. Figures 4 and 5 present the results for analysis of urine samples collected from ten subjects at six-hour and fourteen-day time points, respectively. The highest concentration of 7-aminoflunitrazepam in six-hour urine collection was 518 ng/mL and the lowest 70 ng/mL. In fourteen-day collection the highest 7-aminoflunitrazepam urine concentration was 500 pg/mL (subject #3) and the lowest 40 pg/mL (subject #10). In nine subjects the maximum concentration of 7-aminoflunitrazepam in urine was observed six hours after drug administration and in one subject (volunteer #9) at 24-hour time point. Figure 6 presents results of urine analysis by NCI-GC-MS and micro-plate enzyme immunoassay after enzymatic hydrolysis and solid-phase extraction. In three subjects (subjects #5, #9 and #10) micro-plate enzyme immunoassay gave positive results for benzodiazepines up to 5 days after administration of a single dose of Rohypnol®, in four subjects (#1, #2, #3 and #4) after 8 days, in two subjects (#7 and #8) up to 10 days and in one subject (#4) 21 days after drug administration.

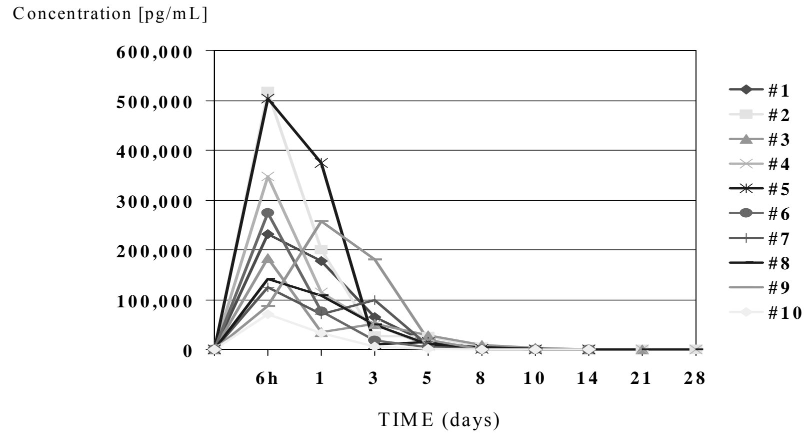

Figure 2. Elimination of 7-aminoflunitrazepam in urine of ten subjects.

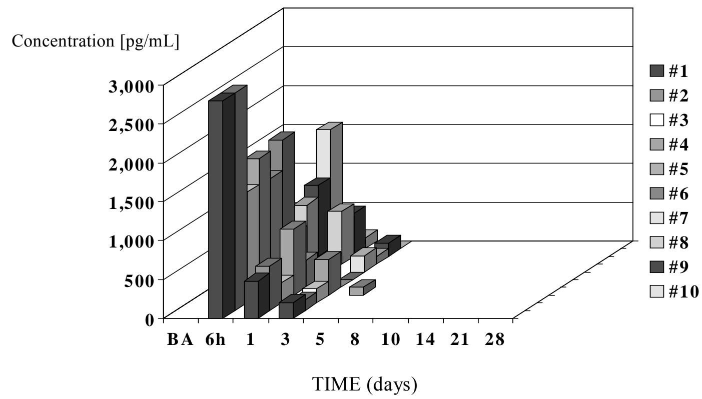

Figure 3. Elimination of flunitrazepam after a single dose of Rohypnol®.

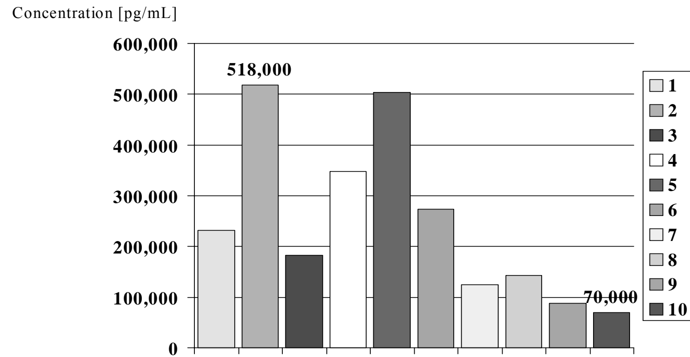

Figure 4. Concentrations of 7-aminoflunitrazepam in urine samples collected from ten volunteers six hours after Rohypnol® administration.

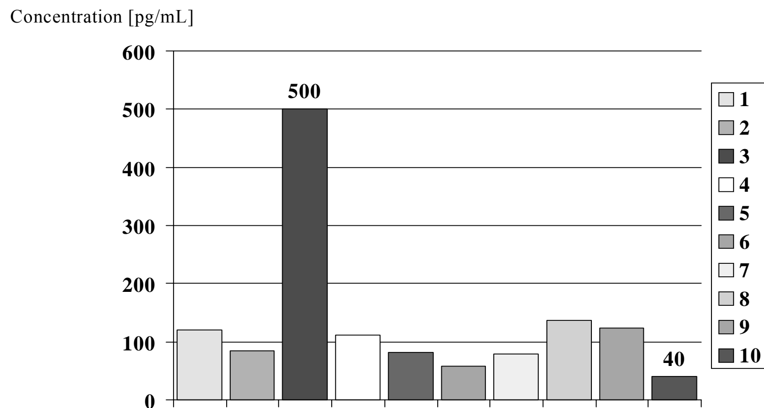

Figure 5. Concentrations of 7-aminoflunitrazepam in urine samples collected from ten volunteers fourteen days after Rohypnol® administration.

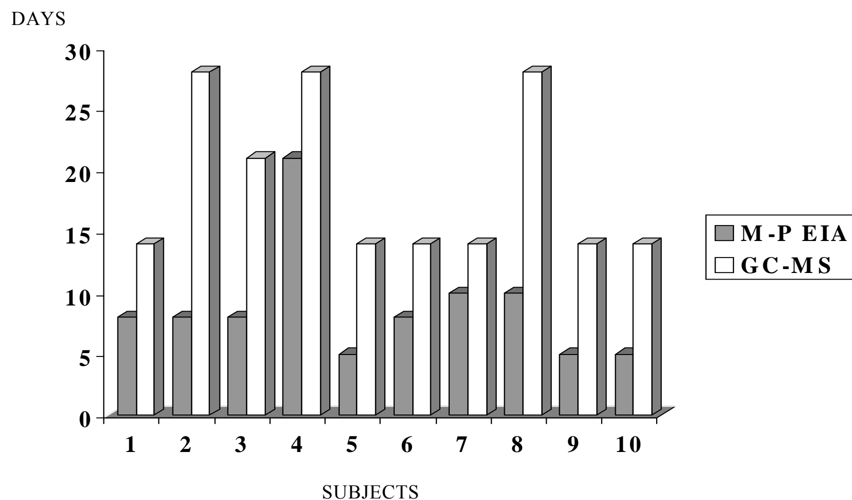

Figure 6. Results of urine analysis by NCI-GC-MS and micro-plate enzyme immunoassay after enzymatic hydrolysis and solid-phase extraction.

Elimination of 7-aminoflunitrazepam in urine collected from 10 volunteers in the second group are presented in Figure 7. Figure 8 shows urine concentrations of flunitrazepam in the same group of subjects. The highest concentrations of 7-aminoflunitrazepam were observed 24 hours after Rohypnol® administration in eight out ten volunteers. In two subject the highest concentration of 7-aminoflunitrazepam was observed 6 hours after administration of flunitrazepam. Figure 9 presents the results for analysis of urine samples collected from ten subjects at fourteen-day time point. In fourteen-day collection the highest 7-aminoflunitrazepam urine concentration was 473 pg/mL (subject #9) and the lowest 23 pg/mL (subject #3).

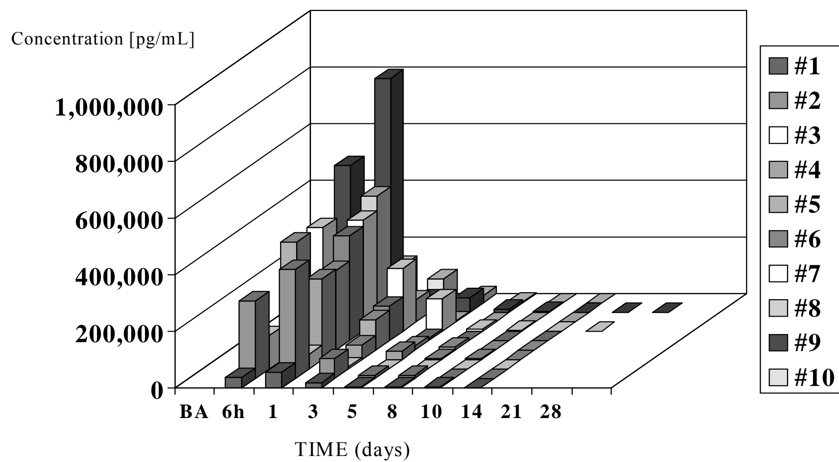

Figure 7. Elimination of 7-aminoflunitrazepam in urine collected from ten volunteers in the second group.

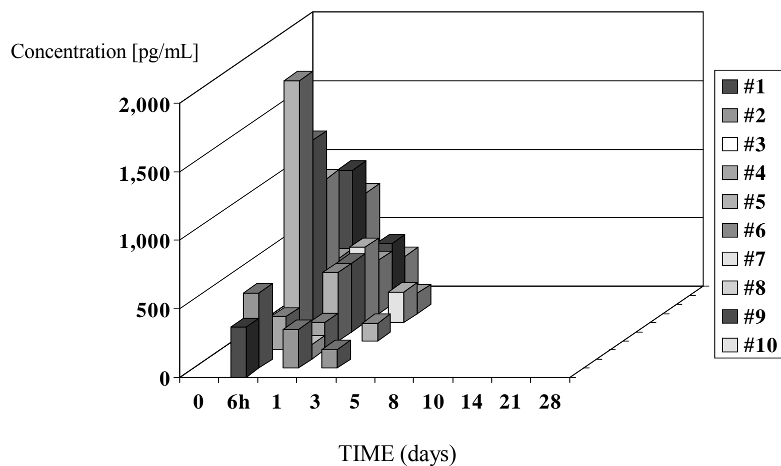

Figure 8. Elimination of flunitrazepam in urine collected from ten volunteers in the second group.

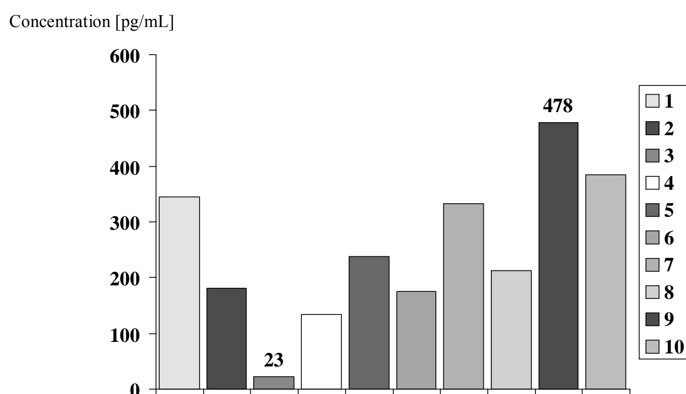

Figure 9. Concentrations of 7-aminoflunitrazepam in urine samples collected from ten volunteers (second group) fourteen days after Rohypnol® administration.

2. <u>Detection of Flunitrazepam and 7-Aminoflunitrazepam in Hair</u>

Subjects and Specimens

Ten volunteer subjects (8 women and 2 men, 21 to 49 years old) were admitted to the Psychiatric Unit of the University of Illinois at Chicago Hospital between July 20 and July 29, 1999. Table 3 shows the age, race, gender, hair color, and hair treatment of the ten participants. One sample was collected from each subject before drug administration. A single 2 mg dose of Rohypnol® was given to each subject at approximately 8:30 AM. The subjects were housed in the Psychiatric Unit of the UIC Hospital for approximately 8 hours following drug administration. The hair samples were collected on the following days after drug administration: 24 hours (day 1), 3, 5, 14, 21, and 28 days. The hair (equivalent to thickness of a pencil) was clipped close to the scalp

from the back of the head and placed in the hair collection kit kindly supplied by the United Drug Testing Laboratories, Inc., with the root end indicated and stored at room temperature until analysis. The single-dose drug clinical study was reviewed and approved by the Food and Drug Administration and the Institutional Review Board of the University of Illinois at Chicago.

Table 3. Subject Information

VOLUNTEER	AGE	RACE	SEX	NATIVE HAIR COLOR	TREATMENT
1	41	White	F	Brown	Semi-permanent color 1/2 weeks ago
2	28	White	F	Brown	No chemicals used
3	22	White	F	Red	Colored 1 year ago
4	47	White	F	Gray	Permed
5	43	White	M	Gray	No chemicals used
6	33	Asian	M	Black	Uses gel
7	21	White	F	Blonde	Dyed 5 weeks ago
8	26	White	F	Brown-Blonde	No chemicals used
9	44	White	F	Gray-Blonde	Permed 3 weeks ago
10	49	African American	F	Black	Permed 6 weeks ago

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 Company, Wilmington, DE). An HP-5MS capillary column (30 m x 250 μm x 0.25 μm) was used for separation (Hewlett Packard Company, Wilmington, DE). The heating block was from Fisher Scientific (Itasca, IL) and vacuum oven model 5831 (Napco®) purchased from Fisher Scientific (Itasca, IL). The hair pulverizer was acquired from Crescent (Lyons, IL). The Vac-Elut™ extraction manifold was from Analytical International (Varian, Harbor City, CA), the centrifuge model 5810 (Eppendorf-Netheler-Hinz GmbH, Germany) was acquired

from Brinkmann Instruments, Inc., Westbury, NY, and the Meyer N-EVAP® analytical evaporator was from Organomation Assoc., Inc. (Northborough, MA). The Aerograph water bath sonicator was purchased from Varian (Harbor City, CA).

Materials and Reagents

Flunitrazepam (1 mg/mL in methanol), 7-aminoflunitrazepam (100 μg/mL in acetonitrile), and the deuterated internal standard D_5 diazepam (100 μg/mL in methanol) were all purchased from Radian International (Austin, TX). Methanol (HPLC grade), hydrochloric acid (certified A.C.S. Plus), glacial acetic acid (HPLC grade), methylene chloride (HPLC/GC/MS grade), isopropanol (HPLC grade), ethyl acetate (HPLC grade) and concentrated ammonium hydroxide (certified A.C.S. Plus) were bought from Fisher Scientific (Itasca, IL). Heptafluorobutyric anhydride (HFBA) was purchased from Campbell Supply Company (Rockton, IL). The HCX Isolute® 10 mL 200 mg columns (International Sorbent Technologies) were purchased from Jones Chromatography (Lakewood, CO).

Standards and Controls

The flunitrazepam (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-aminoflunitrazepam (100 μg/mL in acetonitrile) standard stock solution was diluted to 10 μg/mL, 0.1 μg/mL, and 10 ng/mL. The deuterated internal standard D_5 diazepam (100 μg/mL in methanol) was diluted to 10 μg/mL and 1 μg/mL. All standards were diluted in their respective solvents.

A five-point standard curve was made for 7-aminoflunitrazepam and a four-point curve for flunitrazepam. The concentrations of flunitrazepam in standard hair preparations were as follows: 2.5, 5.0, 10.0, and 15.0 pg/mg of hair. The concentrations of 7-aminoflunitrazepam were: 0.5, 1.0,

5.0, 10.0, and 20.0 pg/mg of hair. In addition, two levels of controls were prepared. Low and high controls were spiked with flunitrazepam and 7-aminoflunitrazepam. The low control (3 pg/mg 7-aminoflunitrazepam and 4 pg/mg flunitrazepam) was prepared by adding 15 µL of the 10 pg/µL 7-aminoflunitrazepam standard stock solution and 20 µL of the 10 pg/µL flunitrazepam standard stock solution to 50 mg of pulverized negative hair. The high control (15 pg/mg 7-aminoflunitrazepam and 12 pg/mg flunitrazepam) was prepared by adding 75 µL of the 10 pg/µL 7-aminoflunitrazepam stock solution and 60 µL of the 10 pg/µL solution of flunitrazepam to 50 mg of pulverized negative hair.

Analytical Procedure

The volunteer subject's hair samples were removed from the envelopes, cut approximately 1.5 cm from the root end, pulverized and 50 mg aliquots were analyzed. To the volunteers' hair samples, standard, and control hair preparations 30 µL of the 1.0 µg/mL solution of the internal standard, D_5 diazepam, was added to reach a final concentration of 600 pg/mg. This was followed by the addition of methanol (3 mL) and sonication for one hour. The tubes were then centrifuged for 5 minutes at 400*g* and the supernatant was transferred to clean tubes and stored at 4°C. To the remaining hair, 0.1 N HCL (3 mL) was added and the hair was digested at 55°C for 18 - 24 hours. The hair samples were again centrifuged (5 minutes at 400*g*), the supernatants were pooled, and 1.93 M 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 1.93 M 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 two minutes. The bed of the column was washed with 3 mL of deionized water (dried

for one to two minutes), 1 mL of 0.1 N hydrochloric acid (dried for one to two minutes) and 3 mL of methanol (dried for five minutes). The collection tubes were placed in the rack and the drugs were eluted using mixture of methylene chloride:isopropanol:ammonium hydroxide (78:20:2 v/v/v) (3 mL). The eluent was evaporated to dryness at room temperature using a stream of nitrogen. The dry residue was reconstituted in ethyl acetate (50 μL) and transferred to autosampler vials. The extract was evaporated to dryness in the vacuum oven at 60^0C. The samples were derivatized using HFBA (50 μL) at 60^0C for 30 minutes in the sealed vials. After incubation, the derivatizing agent was evaporated in the vacuum oven (60^0C) and the dry residue reconstituted in ethyl acetate (25 μL).

Chromatographic Method

The injector was operated in the splitless mode and the injection volume was 1 μL. The injector temperature was 240^0C. Ultra high purity helium (99.999%) was used as the carrier gas at a constant flow of 1.2 mL per minute. The initial GC oven temperature of 60^0C was held for one minute, and then increased at a rate of 30^0C per minute until the final temperature of 310^0C was attained. The final temperature was held for three minutes. The total run time for one injection was 12.33 minutes. The transfer line temperature was maintained at 280^0C. Methane (ultra high purity - 99.999%) was used as reagent gas at an apparent pressure of 3.8×10^{-4} Torr in the ion source (methane flow 3.25 mL/min.) The MS ion source temperature was 250^0C and the quadrupole temperature was 106^0C. The electron multiplier voltage was set at +400V above the NCI-tune voltage.

The mass selective chemical ionization detector was monitoring negative ions (NCI) and it was operating in the selected ion monitoring (SIM) mode. The solvent delay was 9.10 minutes. The following ions were monitored and used for quantitation: for flunitrazepam *m/z* 313 and 297, 7-

aminoflunitrazepam *m/z* 459 and 441, and for D_5 diazepam *m/z* 289. The dwell time for the ions was 20 ms. The liner was frequently changed since flunitrazepam is particularly sensitive to active sites.

Precision and Accuracy

Quantitation of flunitrazepam and 7-aminoflunitrazepam was performed using an internal standard method. A five-point standard curve was prepared by linear least square regression analysis of the ratio of the peak area of 7-aminoflunitrazepam to the peak area of the internal standard (D_5 diazepam) as a function of concentration. A separate four-point standard curve was also prepared for flunitrazepam with D_5 diazepam as the internal standard. Peak area ratios were determined for the controls and the controls were then calculated using the standard curve. The intra-day variability was determined by analyzing six low controls (3 pg/mg 7-aminoflunitrazepam, 4 pg/mg flunitrazepam) and six high controls (15 pg/mg 7-aminoflunitrazepam, 12 pg/mg flunitrazepam) on a single day. The inter-day variability was determined over a period of five weeks. The mean measured concentrations and standard deviations were calculated based on the intra- and inter-day variability populations. The percent coefficient of variation was determined by dividing the standard deviation by the mean measured concentration and multiplying by 100%. The percent relative accuracy was determined using the following equation:

(Mean Measured Concentration - Theoretical Concentration)/Theoretical Concentration] x 100%.

Results

The retention times of flunitrazepam, 7-aminoflunitrazepam, and D_5 diazepam were approximately 9.81, 9.43 and 9.30 minutes, respectively. The chromatograms were recorded over the time range of 9.10 to 10.50 minutes. The standard curves for flunitrazepam and 7-aminoflunitrazepam were linear over the range of concentrations analyzed (2.5 pg/mg to 15 pg/mg

hair for flunitrazepam, and 0.5 pg/mg to 20 pg/mg hair for 7-aminoflunitrazepam). The correlation coefficients of the standard curves were 0.988 and 0.999 for flunitrazepam and 7-aminoflunitrazepam, respectively. The limits of detection were 0.5 pg/mg for flunitrazepam and 0.2 pg/mg for 7-aminoflunitrazepam, which were the lowest concentrations of both drugs at which the signal-to-noise ratio was 3:1. The limits of quantitation were 2.5 pg/mg and 0.5 pg/mg for flunitrazepam and 7-aminoflunitrazepam, respectively. Both limits of quantitation were arbitrarily established to be the lowest concentrations on the corresponding standard curves.

Tables 4 and 5 describe the accuracy and precision of the flunitrazepam and 7-aminoflunitrazepam control preparations, respectively. The intra-day variability was determined by analyzing six low controls (3 pg/mg 7-aminoflunitrazepam, 4 pg/mg flunitrazepam) and six high controls (15 pg/mg 7-aminoflunitrazepam, 12 pg/mg flunitrazepam) on a single day. The inter-day variability was determined over a period of five weeks.

All pre-drug hair specimens were negative for both flunitrazepam and 7-aminoflunitrazepam. The concentrations of flunitrazepam detected in the hair of all ten subjects were below the limit of quantitation (2.5 pg/mg) and only five volunteers had flunitrazepam concentrations higher than the limit of detection (0.5 pg/mg) (subject #1, 0.8 pg/mg on day 3; subject #3, 1.3 pg/mg and 1.5 pg/mg on day 1 and day 3, respectively; subject #5, 1.2 pg/mg on day 1, 1.3 pg/mg on day 3, and 0.5 pg/mg on day 5; subject #7, 1.3 pg mg on day 28; and subject #9, 1.1 pg/mg on day 1, 2.3 pg/mg on day 3, 1.2 pg/mg on day 14, 0.6 pg/mg on day 21).

Table 6 presents the concentrations of 7-aminoflunitrazepam in the subjects' hair samples. 7-Aminoflunitrazepam was detected up to 28 days after Rohypnol® administration in the hair of all ten volunteers. In five volunteers (subjects #1, #3,#4, #5, and #9), 7-aminoflunitrazepam was

detected 24 hours after flunitrazepam administration and remained in the hair throughout the entire 28-day study period (0.6-8.0 pg/mg) (Table 6). In two cases (subjects #6 and #10), 7-aminoflunitrazepam appeared in hair 14 days after drug intake (0.6-5.4 pg/mg), while in two subjects (#2 and #7) it appeared 21 days after administration (0.5-2.7pg/mg) (Table 6). In subject #8, 7-aminoflunitrazepam concentrations were below the quantitation limit. The day on which the highest concentration of 7-aminoflunitrazepam was detected for each subject varied, therefore making it difficult to determine the time frame necessary to achieve the maximum concentration in hair.

The largest amount of 7-aminoflunitrazepam detected was 8 pg/mg on day 28 (subject #9). For subject #1, the highest concentration of 7-aminoflunitrazepam was found on day 3 (5.8 pg/mg). On day 14, the highest concentration of 7-aminoflunitrazepam was found for subjects #3 (6.2 pg/mg), #4 (4.4 pg/mg), and #5 (4.1 pg/mg). On day 28, the highest concentration was found for subjects #2 (2.7 pg/mg), #6 (5.4 pg/mg), #7 (2.2 pg/mg), #9 (8.0 pg/mg), and #10 (1.4 pg/mg). There are three cases (subjects #2, #6, and #7) in which the concentration of 7-aminoflunitrazepam continued to increase with increasing time. Coincidentally, these three subjects also had the latest first date of detection among the ten subjects (21 days, 14 days, and 21 days, respectively). Due to the fact that our study ended at 28 days, it is impossible to determine what would happen to the concentrations after this date.

Table 4. Accuracy and Precision of Flunitrazepam Control Hair Preparations (pg/mg)

Parameter	Low Control	High Control
Target Concentration	4	12
INTRA-DAY	N=6	N=6
Mean Measured Conc. (±S.D.)	3.7 (±0.3)	12.9 (±0.9)
% Coefficient of Variation	8.1	7.0
% Relative Accuracy	-7.5	7.5
INTER-DAY	N=15	N=16
Mean Measured Conc. (±S.D.)	3.4 (±0.4)	12.3 (±0.9)
% Coefficient of Variation	11.8	7.3
% Relative Accuracy	-15.0	2.5

Table 5. Accuracy and Precision of 7-Aminoflunitrazepam Control Hair Preparations (pg/mg)

Parameter	Low Control	High Control
Target Concentration	3	15
INTRA-DAY	N=6	N=6
Mean Measured Conc. (±S.D.)	2.9 (±0.3)	14.6 (±1.1)
% Coefficient of Variation	10.3	7.5
% Relative Accuracy	-3.3	-2.7
INTER-DAY	N=15	N=16
Mean Measured Conc. (±S.D.)	3.8 (±0.7)	15.5 (±2.1)
% Coefficient of Variation	18.4	13.5
% Relative Accuracy	26.6	3.3

Table 6. 7-Aminoflunitrazepam Concentrations in Subjects' Hair (pg/mg)

Subjects	Before dosing	1 day	3 days	5days	14 days	21 days	28 days
1	ND	2.0	5.8	-[1]	4.1	5.1	4.2
2	ND	ND	ND	ND	ND	0.7	2.7
3	ND	5.3	5.2	0.7	6.2	5.5	5.8
4	ND	0.6	1.8	3.1	4.4	4.2	3.2
5	ND	1.8	4.0	2.6	4.1	3.6	2.9
6	ND	ND	ND	ND	0.8	4.4	5.4
7	ND	ND	ND	ND	ND	0.5	2.2
8	ND	ND	ND	ND	0.4[2]	0.4[2]	ND
9	ND	2.2	7.4	-[1]	5.0	6.4	8.0
10	ND	ND	ND	ND	0.6	0.3[2]	1.4

[1]Missing sample
[2]Below quantitation limit
ND - not detected

II Detection of Clonazepam and 7-Aminoclonazepam in Hair

Subjects and Specimens

Ten subjects (4 women and 6 men, age 26 to 66 years old) who were already being treated with clonazepam 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 of 1999 using hair collection kits provided by the United States Drug Testing Laboratories, Inc. A single hair sample (equivalent to 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 (IRB number H-98-1096).

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 x 250 µm x 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 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, Inc., Westbury, NY,and the Meyer N-EVAP® analytical evaporator from Organomation Assoc., Inc. (Northborough, MA). The Aerograph water bath sonicator was purchased from Varian (Harbor City, CA).

Chemicals and Reagents

Clonazepam (1 mg/mL in methanol), 7-aminoclonazepam (100 μg/mL in acetonitrile), and the deuterated internal standard D_5 diazepam (100 μg/mL in methanol) were purchased from Radian International (Austin, TX). Methanol (HPLC grade), hydrochloric acid (certified A.C.S. Plus), glacial acetic acid (HPLC grade), methylene chloride (HPLC/GC/MS grade), isopropanol (HPLC grade), ethyl acetate (HPLC grade) and concentrated ammonium hydroxide (certified A.C.S. Plus) were bought from Fisher Scientific (Itasca, IL). Heptafluorobutyric anhydride (HFBA) was purchased from Campbell Supply Company (Rockton, IL). The HCX Isolute® 200 mg, 10 mL columns (International Sorbent Technologies) were purchased from Jones Chromatography (Lakewood, CO).

Standards and Controls

The clonazepam (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-aminoclonazepam (100 μg/mL in acetonitrile) standard stock solution was diluted to 10 μg/mL, 1 μg/mL, 0.1 μg/mL, and 10 ng/mL. The deuterated internal standard, D_5 diazepam (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-aminoclonazepam and a five-point curve for clonazepam by using 50 mg aliquots of the negative pulverized hair spiked with solutions of both drugs. The concentrations of the 7-aminoclonazepam in standard hair preparations were as follows: 1, 5, 10, 50, 100, 200, 500, and 1000 pg/mg of hair. The concentrations of clonazepam were 10, 50, 100, 200, and 400 pg/mg of hair. In addition, two levels of controls were prepared. The low controls for 7-aminoclonazepam (3 pg/mg) and clonazepam (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-aminoclonazepam (800 pg/mg) and clonazepam (300 pg/mg) were prepared by adding 40 μL of 1000 pg/μL and 75 μL of 200 pg/μL, respectively.

Analytical Procedure

The hair samples were divided into approximately 2 cm segments, 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 D_5 diazepam (600 pg/mg) was added. Methanol (3 mL) was added to the hair and it was sonicated for 1 hour. The samples were centrifuged for five minutes at 400*g,* the methanol layer was decanted, and transferred to clean test tubes for storage in the refrigerator. To the remaining hair, 0.1 N hydrochloric acid (3 mL) was added and the specimens were incubated overnight at 55^0C, after being sealed and vortexed. The test tubes were removed from the heating block and centrifuged for five minutes at 400*g*. The methanol and acid were combined, with the addition of 1.93 M glacial acetic acid (1 mL) and deionized water (9 mL). 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 1.93 M 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 two minutes. The bed of the column was washed with 3 mL of deionized water (dried for one to two minutes), 1 mL of 0.1 N hydrochloric acid (dried for one to two minutes) and 3 mL of methanol (dried for five minutes). The collection tubes were placed in the rack and the drugs were eluted using 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 60^0C. The samples were derivatized using 50 µL of HFBA at 60^0C for 30 minutes in the sealed vials. After incubation, the derivatizing agent was evaporated in the vacuum oven at 60^0C and the dry residue reconstituted in 25 µL of ethyl acetate.

Chromatographic Method

The injector was operated in the splitless mode at 240^0C, 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 per minute. The initial GC oven temperature of 60^0C was held for one minute, and then increased at a rate of 30^0C per minute until the final temperature of 310^0C was attained. The final temperature was held for three minutes. The total run time for one injection was 12.33 minutes. Methane (ultra high purity - 99.999%) was used as reagent gas at an apparent pressure of 3.7×10^{-4} Torr in the ion source (methane flow 3.25 mL/min.) The MS ion source temperature was 250^0C and the quadrupole temperature was 106^0C. 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 nine minutes. The detector was turned off after 11.1 minutes. The following ions were monitored and used for quantitation: for clonazepam *m/z* 315 and 279, for 7-aminoclonazepam *m/z* 461 and 445, and for for D_5 diazepam *m/z* 289. 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 since clonazepam is particularly sensitive to active sites.

Precision and Accuracy

Quantitation of clonazepam and 7-aminoclonazepam was performed by the internal standard method. An eight-point standard curve for 7-aminoclonazepam and five-point standard curve for clonazepam was prepared by linear least square regression analysis of the ratio of the peak area of 7-aminoclonazepam and clonazepam to the peak area of the internal standard, D_5 diazepam. Peak area ratios were determined for the control hair preparations. Control concentrations were calculated from the standard curve values.

Intra-day 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 clonazepam and 7-aminoclonazepam, respectively. Inter-day variability was ascertained over a period of eight weeks. The mean measured concentrations and standard deviations were calculated based on the inter- and intra-day variability populations.

The percent relative accuracy was calculated by the following equation: [(Mean Measured Concentration - Theoretical Concentration) / Theoretical Concentration] x 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 time of 7-aminoclonazepam was approximately 10.10 minutes, clonazepam was 10.77 minutes, and D_5 diazepam was 9.37 minutes. All chromatograms were recorded over the time range of 9.00 to 11.10 minutes. The standard curves were linear over the range of drugs assayed (1 pg/mg to 1000 pg/mg for 7-aminoclonazepam and 10 pg/mg to 400 pg/mg for clonazepam) and had correlation coefficients of 0.998. The limit of quantitation for 7-

aminoclonazepam was 1 pg/mg and for clonazepam 10 pg/mg for 50 mg sample. Both limits of quantitation were arbitrarily established to be the lowest concentrations on the corresponding standard curves. The limit of detection for clonazepam was 5 pg/mg and for 7-aminoclonazepam 0.4 pg/mg, which was the lowest concentrations of drugs at which the signal-to-noise ratio was 3:1. Tables 7 and 8 present the accuracy and precision of the clonazepam and 7-aminoclonazepam control hair preparations, respectively. The intra-day variability was determined by analyzing three low and four high replicates of controls (30 and 300 pg/mg of hair for clonazepam and 3 and 800 pg/mg of hair for 7-aminoclonazepam) prepared in hair on a single day. The inter-day variability was determined over a period of eight weeks on 10 separate days.

Figure 10 presents concentrations of 7-aminoclonazepam in hair samples collected from subjects #1 and #2. Both subjects received a single 2 mg dose of clonazepam approximately three weeks before hair collection. For subject #1 only traces (below quantitation limit) of 7-aminoclonazepam were detected; for subject #2 4.8 pg/mg of 7-aminoclonazepam were detected. The concentrations of 7-aminoclonazepam in remaining eight hair samples are presented in Figure 11. 7-Aminoclonazepam was present in measurable quantities in 9 out of 10 hair samples collected and the concentration range was 1.37 - 1,267 pg/mg. Figure 12 shows concentrations of parent drug, clonazepam, in study samples. It was not detected in only 4 out of 10 samples and the concentration range was 10.7 - 180.6 pg/mg.

Table 7. Accuracy and Precision of Clonazepam Hair Preparations (pg/mg)

Parameter	Low Control	High Control
Target Concentration	30	300
INTRA-DAY	N=3	N=4
Mean Measured Conc. (±S.D.)	28.99 (±2.14)	288.81 (±46.20)
% Coefficient of Variation	7.38	16.00
% Relative Accuracy	-3.37	-3.73
INTER-DAY	N=31	N=29
Mean Measured Conc. (±S.D.)	31.88 (±9.06)	291.49 (±73.40)
% Coefficient of Variation	28.42	25.18
% Relative Accuracy	6.27	-2.84

Table 8. Accuracy and Precision of 7-Aminoclonazepam Hair Preparations (pg/mg)

Parameter	**Low Control**	**High Control**
Target Concentration	3	800
INTRA-DAY	N=3	N=4
Mean Measured Conc. (±S.D.)	3.23 (±0.31)	805.96 (±63.75)
% Coefficient of Variation	9.60	7.91
% Relative Accuracy	7.67	0.74
INTER-DAY	N=30	N=23
Mean Measured Conc. (±S.D.)	3.24 (±0.79)	836.73(±138.84)
% Coefficient of Variation	24.45	16.59
% Relative Accuracy	7.67	4.59

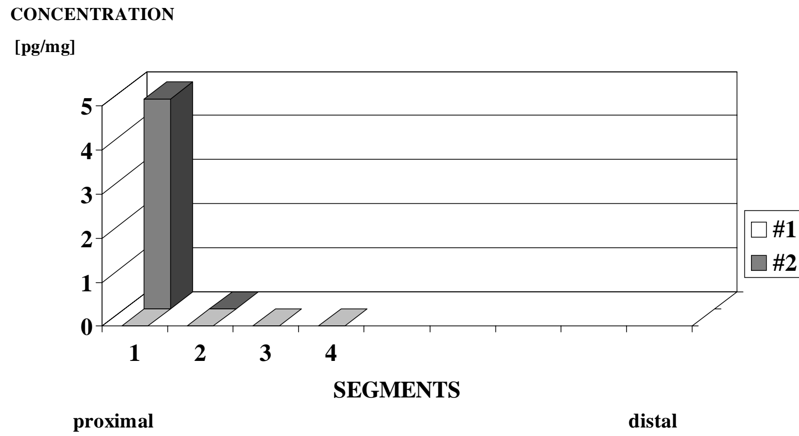

Figure 10. Concentrations of 7-aminoclonazepam in hair samples collected from subjects #1 and #2.

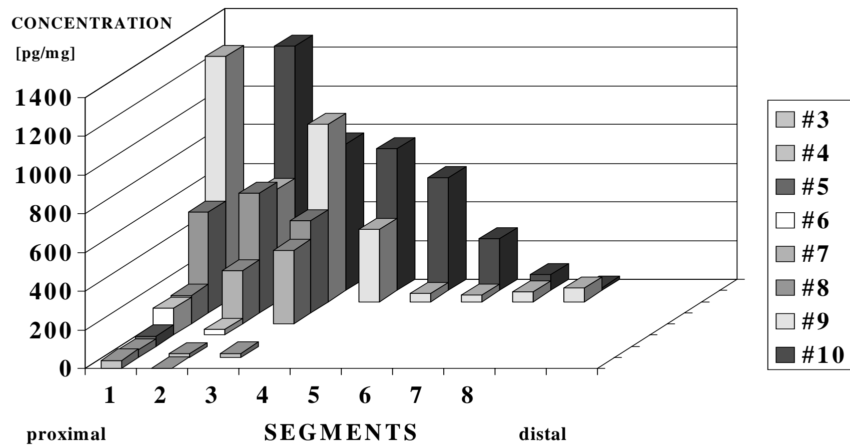

Figure 11. Concentrations of 7-aminoclonazepam in hair of remaining eight patients.

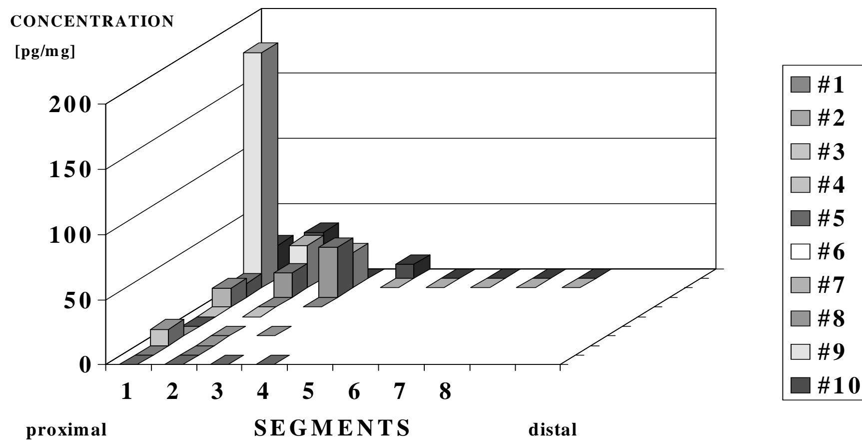

Figure 12. Concentrations of clonazepam in study samples.

III Detection of Diazepam, Alprazolam, and Triazolam in Hair

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 x 250 µm x 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 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, Inc., Westbury, NY,and the Meyer N-EVAP® analytical evaporator from Organomation Assoc., Inc. (Northborough, MA). The Aerograph water bath sonicator was purchased from Varian (Harbor City, CA).

Chemicals and Reagents

Diazepam (1 mg/mL in methanol), alprazolam (1mg/mL in methanol), triazolam (1mg/mL in methanol) and the deuterated internal standard D_5 diazepam (100 μg/mL in methanol) were purchased from Radian International (Austin, TX). Methanol (HPLC grade), hydrochloric acid (certified A.C.S. Plus), glacial acetic acid (HPLC grade), methylene chloride (HPLC/GC/MS grade), isopropanol (HPLC grade), ethyl acetate (HPLC grade) and concentrated ammonium hydroxide (certified A.C.S. Plus) were bought from Fisher Scientific (Itasca, IL). The HCX Isolute® 200 mg, 10 mL columns (International Sorbent Technologies) were purchased from Jones Chromatography (Lakewood, CO).

Standards and Controls

The diazepam, alprazolam and triazolam (1 mg/mL in methanol) standard stock solutions were diluted to 100 μg/mL, 1 μg/mL, and 10 ng/mL . The deuterated internal standard, D_5 diazepam (100 μg/mL in methanol), was diluted to 10 μg/mL and 1 μg/mL. All standards were diluted in their respective solvents.

A five-point standard curve was made for each compound by using 50 mg aliquots of the negative pulverized hair spiked with solutions of all drugs. The concentrations of diazepam were 10, 20, 30, 40, and 50 pg/mg of hair. The concentrations of alprazolam and triazolam were 2.5, 5, 10, 15, and 20 pg/mg of hair. In addition, two levels of controls were prepared. The low control for diazepam was 25 pg/mg, for alprazolam and triazolam was 7 pg/mg. The high controls for

alprazolam and trazolam were 16 pg/mg and for diazepam 42 pg/mg.

Analytical Procedure

To each standard and control preparations, 30 µL of 1 µg/mL of D_5 diazepam (600 pg/mg) was added. Methanol (3 mL) was added to the hair and it was sonicated for 1 hour. The samples were centrifuged for five minutes at 400*g*, the methanol layer was decanted, and transferred to clean test tubes for storage in the refrigerator. To the remaining hair, 0.1 N hydrochloric acid (3 mL) was added and the specimens were incubated overnight at 55^0C, after being sealed and vortexed. The test tubes were removed from the heating block and centrifuged for five minutes at 400*g*. The methanol and acid were combined, with the addition of 1.93 M glacial acetic acid (1 mL) and deionized water (9 mL). 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 1.93 M 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 two minutes. The bed of the column was washed with 3 mL of deionized water (dried for one to two minutes), 1 mL of 0.1 N hydrochloric acid (dried for one to two minutes) and 3 mL of methanol (dried for five minutes). The collection tubes were placed in the rack and the drugs were eluted using 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 30 µL of ethyl acetate and transferred to autosampler vials.

Chromatographic Method

The injector was operated in the splitless mode at 240^0C, 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 per minute. The initial GC oven temperature of 60^0C was held for one minute, and then increased at a rate of 30^0C per minute until the final temperature of 310^0C was attained. The final temperature was held for three minutes. The total run time for one injection was 12.33 minutes. Methane (ultra high purity - 99.999%) was used as reagent gas at an apparent pressure of 3.7×10^{-4} Torr in the ion source (methane flow 3.25 mL/min.) The MS ion source temperature was 250^0C and the quadrupole temperature was 106^0C. 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 nine minutes. The detector was turned off after 11.1 minutes. The following ions were monitored and used for quantitation: for diazepam *m/z* 284 and 254 , for alprazolam *m/z* 308 and 310, for triazolam *m/z* 306 and 308 and for for D_5 diazepam *m/z* 289. The dwell time for all ions was 20 ms. The single-taper deactivated liners with glass wool were used.

Precision and Accuracy

Quantitation of triazolam, diazepam and alprazolam was performed by the internal standard method. A five-point standard curves for all drugs were prepared by linear least square regression analysis of the ratio of the peak area of triazolam, alprazolam, and diazepam to the peak area of the internal standard, D_5 diazepam. Peak area ratios were determined for the control hair preparations. Control concentrations were calculated from the standard curve values.

Intra-day variability was ascertained by analyzing six replicates of low controls for each drug Inter-day variability was ascertained over a period of four weeks. The mean measured concentrations and standard deviations were calculated based on the inter- and intra-day variability populations.

The percent relative accuracy was calculated by the following equation: [(Mean Measured Concentration - Theoretical Concentration) / Theoretical Concentration] x 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 time of diazepam and D_5 diazepam was approximately 9.3 minutes, alprazolam was 11 minutes, and triazolam 11.5 minutes. All chromatograms were recorded over the time range of 9.00 to 12.10 minutes. The standard curves were linear over the range of drugs assayed (2.5 pg/mg to 20 pg/mg for alprazolam and triazolam and 10 pg/mg to 50 pg/mg for diazepam) and had correlation coefficients of 0.987, 0.998 and 0.992, respectively. The limit of quantitation for alprazolam and triazolam was 2.5 pg/mg and for diazepam 10 pg/mg for 50 mg sample. All limits of quantitation were arbitrarily established to be the lowest concentrations on the corresponding standard curves. Tables 9, 10 and 11 present the accuracy and precision of the alprazolam, triazolam and diazepam control hair preparations, respectively.

Table 9. Accuracy and Precision of Alprazolam Hair Preparations (pg/mg)

Parameter	Low Control	High Control
Target Concentration	7	16
INTRA-DAY	N=6	N=6
Mean Measured Conc. (±S.D.)	7.5 (±0.8)	16.6 (±1.8)
% Coefficient of Variation	10.7	10.8
% Relative Accuracy	7.1	3.7
INTER-DAY	N=16	N=19
Mean Measured Conc. (±S.D.)	6.8 (±1.0)	15.6 (±1.6)
% Coefficient of Variation	14.7	10.2
% Relative Accuracy	-2.8	-2.5

Table 10. Accuracy and Precision of Triazolam Hair Preparations (pg/mg)

Parameter	Low Control	High Control
Target Concentration	7	16
INTRA-DAY	N=6	N=6
Mean Measured Conc. (±S.D.)	8.4 (±0.5)	16.6 (±2.1)
% Coefficient of Variation	5.9	12.6
% Relative Accuracy	20.0	3.7
INTER-DAY	N=19	N=19
Mean Measured Conc. (±S.D.)	6.8 (±1.0)	16.5 (±1.8)
% Coefficient of Variation	20.6	10.9
% Relative Accuracy	-2.8	3.1

Table 11. Accuracy and Precision of Diazepam Hair Preparations (pg/mg)

Parameter	**Low Control**	**High Control**
Target Concentration	25	42
INTRA-DAY	N=6	N=6
Mean Measured Conc. (±S.D.)	26.8 (±3.2)	42.1 (±4.7)
% Coefficient of Variation	11.9	11.1
% Relative Accuracy	7.2	0.2
INTER-DAY	N=18	N=20
Mean Measured Conc. (±S.D.)	26.5 (±3.3)	42.4 (±3.9)
% Coefficient of Variation	12.4	9.2
% Relative Accuracy	6.0	0.9

IV Analytical Method for Detection of Ketamine in Hair

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 x 250 µm x 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 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, Inc., Westbury, NY,and the Meyer N-EVAP® analytical evaporator from Organomation Assoc., Inc. (Northborough, MA). The Aerograph water bath sonicator was purchased from Varian (Harbor City, CA).

Chemicals and Reagents

Ketamine (1 mg/mL in methanol) and the deuterated internal standard D_5 diazepam (100 µg/mL in methanol) were purchased from Radian International (Austin, TX). Methanol (HPLC grade), hydrochloric acid (certified A.C.S. Plus), glacial acetic acid (HPLC grade), methylene chloride (HPLC/GC/MS grade), isopropanol (HPLC grade), ethyl acetate (HPLC grade) and concentrated ammonium hydroxide (certified A.C.S. Plus) were bought from Fisher Scientific (Itasca, IL). The HCX Isolute® 200 mg, 10 mL columns (International Sorbent Technologies) were purchased from Jones Chromatography (Lakewood, CO).

Standards and Controls

The ketamine (1 mg/mL in methanol) standard stock solution was diluted to 100 µg/mL, 1 µg/mL, and 10 ng/mL . The deuterated internal standard, D_5 diazepam (100 µg/mL in methanol), was diluted to 10 µg/mL and 1 µg/mL. All standards were diluted in their respective solvents.

A five-point standard curve was made for ketamine by using 50 mg aliquots of the negative pulverized hair spiked with solutions of all drugs. The concentrations of the drug were 20, 50, 100, 300, and 500 pg/mg of hair. In addition, two levels of controls were prepared. The low control for ketamine was 75 pg/mg. The high control was 400 pg/mg.

Analytical Procedure

To each standard and control preparations, 30 µL of 1 µg/mL of D_5 diazepam (600 pg/mg) was added. Methanol (3 mL) was added to the hair and it was sonicated for 1 hour. The samples were centrifuged for five minutes at 400*g,* the methanol layer was decanted, and transferred to clean test tubes for storage in the refrigerator. To the remaining hair, 0.1 N hydrochloric acid (3 mL) was added and the specimens were incubated overnight at 55^0C, after being sealed and vortexed. The test tubes were removed from the heating block and centrifuged for five minutes at 400*g*. The methanol and acid were combined, with the addition of 1.93 M glacial acetic acid (1 mL) and deionized water (9 mL). 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 1.93 M 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 two minutes. The bed of the column was washed with 3 mL of deionized water (dried for one to two minutes), 1 mL of 0.1 N hydrochloric acid (dried for one to two minutes) and 3 mL of methanol (dried for five minutes). The

collection tubes were placed in the rack and the drugs were eluted using 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 30 μL of ethyl acetate and transferred to autosampler vials.

Chromatographic Method

The injector was operated in the splitless mode at 240^0C, 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 per minute. The initial GC oven temperature of 60^0C was held for one minute, and then increased at a rate of 30^0C per minute until the final temperature of 310^0C was attained. The final temperature was held for three minutes. The total run time for one injection was 12.33 minutes. The MS ion source temperature was 230^0C and the quadrupole temperature was 140^0C.

The mass selective detector was working in the electron ionization mode (EI) and was monitoring selected ions (SIM). The solvent delay was seven minutes. The detector was turned off after 9.5 minutes. The following ions were monitored and used for quantitation: for ketamine *m/z* 180 (used for quantitation), 182 and 209, for D_5 diazepam *m/z* 289. The dwell time for all ions was 20 ms. The single-taper deactivated liners with glass wool were used.

Precision and Accuracy

Quantitation of ketamine was performed by the internal standard method. A five-point standard curve for ketamine was prepared by linear least square regression analysis of the ratio of the peak area of ketamine to the peak area of the internal standard, D_5 diazepam. Peak area ratios were determined for the control hair preparations. Control concentrations were calculated from the

standard curve values.

Intra-day variability was ascertained by analyzing four replicates of low and high controls. Inter-day variability was ascertained over a period of two weeks. The mean measured concentrations and standard deviations were calculated based on the inter- and intra-day variability populations.

The percent relative accuracy was calculated by the following equation: [(Mean Measured Concentration - Theoretical Concentration) / Theoretical Concentration] x 100%. All data were acquired and analyzed by HP software, Enhanced G1701BA ChemStation ver B.00.00 for Windows NT ver 4.0.

Detection of Ketamine in Hair of Monkeys (Stumptail Macaques)

Hair samples collected from 8 monkeys which received ketamine several times were analyzed to study the feasibility of detection of this drug in hair. All samples were pulverized and analyzed according to the procedure described above.

Results

The retention time of D_5 diazepam was approximately 9.3 minutes, and for ketamine was 7.4 minutes. All chromatograms were recorded over the time range of 7.0 to 9.5 minutes. The standard curve was linear over the range of drugs assayed (20 pg/mg to 500 pg/mg and had correlation coefficient of 0.988. The limit of quantitation for ketamine was arbitrarily established to be the lowest concentrations on the standard curve (20 pg/mg). Table 12 presents the accuracy and precision of the ketamine control hair preparations.

All results of analysis of hair samples collected from monkeys are presented in Table 13. As it can be seen, the animals from the social colony (sc) which received ketamine in significantly lower doses had, in general, lower concentration of the drug in hair. A study with a single dose of

ketamine administered to the animal previously not exposed to the drug is currently ongoing. Table 14 summarizes the analytical details for the developed methods of detection of selected drugs in hair.

Table 12. Accuracy and Precision of Ketamine Hair Preparations (pg/mg)

Parameter	Low Control	High Control
Target Concentration	75	400
INTRA-DAY	N=4	N=4
Mean Measured Conc. (±S.D.)	80.4 (±3.2)	362.7 (±5.1)
% Coefficient of Variation	4.0	1.4
% Relative Accuracy	7.2	-9.3
INTER-DAY	N=10	N=10
Mean Measured Conc. (±S.D.)	75.7 (±5.3)	355.4 (±9.1)
% Coefficient of Variation	7.0	2.6
% Relative Accuracy	0.9	-11.1

Table 13. Detection of Ketamine in hair of Monkeys (*Stumptail Macaques*)

ANIMAL	**WEIGHT [kg]**	**DOSE**	**CONC. (pg/mg)**
1 (F[1])	10	10mg/Mo/10Mo	2869
2 (M[2])	13	13mg/Mo/12Mo	3771
3 (F)	12	12mg/Mo/12Mo	6608
4 (F_{sc})	16	16mg/2x/6Mo	1508
5 (F_{sc})	18	18mg/2x/6Mo	2713
6 (F_{sc})	21	21mg/2x/6Mo	738
7 (F_{sc})	9	9mg/2x/6Mo	1272
8 (F_{sc})	7	7mg/3x/9Mo	1369

[1] female,
[2] male,
sc – social colony

Table 14. Detection of Selected Drugs in Hair

DRUG	**LOQ (pg/mg)**	**IONS (*m/z*)**
Flunitrazepam	2.5	*313, 297*
7-Aminoflunitrazepam	0.5	*459, 441*
Clonazepam	10	*315, 279*
7-Aminoclonazepam	1	*461, 445*
Alprazolam	2.5	*308, 310*
Triazolam	2.5	*306, 308*
Diazepam	10	*284, 254*
Ketamine	20	*180, 182, 209* (EI)

V GHB and Scopolamine

Under current award, we were unable to develop an analytical methodology sensitive enough to detect gamma hydroxy butyrate in hair. In addition, gamma hydroxy butyrate is an endogenous substance and therefore the interpretation of the results of hair analysis for this compound could potentially lead to false positive results. In our opinion it is not feasible to detect gamma hydroxy butyrate in hair due to its acidic character. More subsequent research is necessary in this area.

The involvement of scopolamine, a naturally occurring alcaloid, in drug-facilitated sexual assault has not been well documented. In addition, scopolamine is analytically very difficult and we did not have enough time and funds to pursue this issue. Together with GHB it should be included in the future research in this area.

SUMMARY OF THE MAJOR FINDINGS

1. Micro-plate enzyme immunoassay method allows to detect flunitrazepam and related compounds in urine at least up to 5 days after administration of a single dose of Rohypnol® (like in drug-facilitated sexual assault scenarios). This significant increase in the detection time interval is possible if the enzymatic hydrolysis of urine and solid-phase extraction are applied. We recommend micro-plate enzyme immunoassay as a preliminary screening technique of urine in toxicological investigation of drug-facilitated sexual assault when use of benzodiazepines is expected.
2. Application of solid-phase extraction and highly sensitive gas chromatography - mass spectrometry with negative ion monitoring chemical ionization allows to detect 7-aminoflunitrazepam, flunitrazepam major metabolite, in urine 14 days after administration of a single dose of Rohypnol®. The maximum concentration of 7-aminoflunitrazepam in urine was observed 6 to 24 hours after administration of a single dose of Rohypnol®.
3. The concentrations of 7-aminoflunitrazepam in hair are much higher than concentrations of the parent drug, flunitrazepam. The metabolite remains in hair for at least one month after administration of a single dose of Rohypnol®. There is no correlation between dose of Rohypnol® and concentration of both compounds in hair.
4. We conclude that the 7-aminoclonazepam is being deposited in hair in much higher quantities than the parent drug and remains there for extended periods of time.
5. The new technology allows to detect the other compounds such as diazepam, alprazolam, triazolam, and ketamine in hair in very low concentrations.