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Deposition of 7-Aminoflunitrazepam and Flunitrazepam in Hair After a Single Dose of Rohypnol®*

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ABSTRACT: In recent years, there has been a notable increase in the number of reports on drug-facilitated sexual assault. Benzodiazepines are the most common so-called "date-rape" drugs, with flunitrazepam (Rohypnol®) being one of the most frequently mentioned. The aim of this study was to determine whether flunitrazepam and its major metabolite 7-aminoflunitrazepam could be detected in hair collected from ten healthy volunteers after receiving a single 2 mg dose of Rohypnol® using solid phase extraction and NCI-GC-MS. Such data would be of great importance to law enforcement agencies trying to determine the best time interval for hair collection from a victim of drug-facilitated sexual assault in order to reveal drug use. Ten healthy volunteers (eight women and two men, 21 to 49 years old) participated in the study. The following hair samples were collected from each volunteer: one before flunitrazepam administration, and 1, 3, 5, 14, 21, and 28 days after. In five volunteers, 7-aminoflunitrazepam was detected 24 h after flunitrazepam administration and remained in hair throughout the entire 28-day study period (0.6–8.0 pg/mg). In two cases, 7-aminoflunitrazepam appeared in hair 21 days after drug intake (0.5–2.7 pg/mg), and in two subjects 14 days later (0.5–5.4 pg/mg). In one volunteer, 7-aminoflunitrazepam was detected on day 14 and 21 but concentrations were below the quantitation limit. Flunitrazepam was detected in some samples but all concentrations were below the quantitation limit (0.5–2.3 pg/mg).

KEYWORDS: forensic science, forensic toxicology, drug-facilitated sexual assault, 7-aminoflunitrazepam, flunitrazepam, hair analysis, solid phase extraction, NCI - Gas chromatography mass spectrometry

In recent 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 reports on the subject has been observed (1–5). In 1998, there were 95 770 reported rapes in the United States according to FBI Uniform Crime Report Data as summarized in the Statistical Abstract of the United States (6). The fraction of these incidents that may have been facilitated by the clandestine administration of drugs to the victim is unknown. The only relevant data were published by El-Sohly et al. (3) and they are based on analysis of over 1000 urine specimens from a random collection of cases submitted by forensic science/toxicology labs nationwide, specifically in order to screen for and identify alcohol, "date-rape," and other drugs. About 8.2% of the specimens had confirmed benzodiazepines of some kind. "Date-rape" drugs are known to induce sedation and amnesia in the victim, particularly when taken in combination with ethanol.

Flunitrazepam (Rohypnol®-Roche) [5-(2-fluorophenyl)-1,3,dihydro-1-methyl-7-nitro-2*H*-1,4-benzodiazepin-2-one)] belongs to the 7-nitro group of benzodiazepines (7) and its hypnotic effect predominates over the sedative, anxiolytic and muscle-relaxing effects of other compounds from the same pharmacological group. In the human body, flunitrazepam is metabolized almost completely by the liver to 7-aminoflunitrazepam and then to the N-glucuronide, to the N-demethyl metabolite, hydrolysis to the 3-OH metabolite and then to the O-glucuronide (7–9). In the early 1990s flunitrazepam was identified as the drug of choice for the purpose of "drugging" unsuspecting victims and sexually assaulting them while they are under the influence of this substance (1,2,10). There is some indication that flunitrazepam is being used recreationally (11) but whether the drug is taken knowingly or unknowingly, sexual assault while under the influence of flunitrazepam is considered drug-facilitated rape and can be prosecuted as such. As of 1996, the prescription, sale, and importation of flunitrazepam into the U.S. has been banned. Additionally, the "Drug-Induced Rape Prevention and Punishment Act of 1996" was created, punishing the person who with criminal intent distributes a controlled substance to an individual without that individual's knowledge, with up to 20 years in prison.

An unfortunate frustration surrounding cases of drug-facilitated rape is that victims frequently do not report the crime for days to weeks after the alleged event because of a combination of emotions characteristic of sexual assault victims including embarrassment, fear, rejection, denial, mistrust in authority, and most often the amnesia caused by the drug. Typical toxicological tests on blood (12) and urine (9) can generally only detect flunitrazepam for up to 72 h after ingestion due to quick metabolism and elimi-

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nation. In these cases, it can be difficult, if not impossible, to prosecute a suspect. As early as 1994, law enforcement and rape-crisis center officials had identified a need for more sophisticated testing of suspected rape victims to determine if drugs or other substances may have been involved in the sexual assault (3). Several forensically relevant benzodiazepines have been previously identified in plasma (13), urine (14), and hair (15–19) using negative ion chemical ionization gas chromatography - mass spectrometry (NCI-GC-MS). In the recent controlled clinical study Negrusz et al. (20) was able to detect 7-aminoflunitrazepam in urine for extended periods of time (14–28 days) using solid phase extraction and NCI-GC-MS. It is well known, 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 in urine or in blood. Some time ago LeBeau et al. (2) suggested that hair may be a valuable specimen in the cases of drug-facilitated sexual assault when reporting of the crime was delayed. Negrusz et al. (21) described solid phase extraction followed by NCI-GC-MS quantification of flunitrazepam and 7-aminoflunitrazepam in hair and applied it for the first time to the hair samples collected from the victims of drug-facilitated sexual assault.

The main goal of this study was to determine whether flunitrazepam and its major metabolite 7-aminoflunitrazepam could be detected in hair collected from ten heathy volunteers who received a single 2 mg dose of Rohypnol® using a modification of a previously developed analytical method (21). The data would be of great importance to law enforcement agencies trying to determine the best time interval for hair collection from a victim of drug-facilitated sexual assault in order to reveal drug use. To our knowledge this is the first report on clinical studies on deposition of flunitrazepam and its major metabolite in human hair after a single dose of the drug in which the amount of flunitrazepam administered was regulated, thus eliminating some of the inconsistencies that arise when studying chronic drug users.

Methods

Subjects and Specimens

Ten volunteer subjects (eight women and two 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 1 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 h following drug administration. The hair samples were collected on the following days after drug administration: 24 h (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 States 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.

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 $\times$ 250 μm $\times$ 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).

TABLE 1—*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

Standards and Controls

The flunitrazepam (1 mg/mL in methanol) standard stock solution was diluted to 100, 10, 1, and 0.2 μg/mL. The 7-aminoflunitrazepam (100 μg/mL in acetonitrile) standard stock solution was diluted to 10, 0.1 and 10 ng/mL. The deuterated internal standard D_5 diazepam (100 μg/mL in methanol) was diluted to 10 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 subjects' 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 1 h. The tubes were then centrifuged for 5 min 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 to 24 h. The hair samples were again centrifuged (5 min 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 2 min. The bed of the column was washed with 3 mL of deionized water (dried for 1 to 2 min), 1 mL of 0.1 N hydrochloric acid (dried for 1 to 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 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°C. The samples were derivatized using HFBA (50 μL) at 60°C for 30 min in the sealed vials. After incubation, the derivatizing agent was evaporated in the vacuum oven (60°C) 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°C. 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°C was held for 1 min, and then increased at a rate of 30°C per minute 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. The transfer line temperature was maintained at 280°C. 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°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) and it was operating in the selected ion monitoring (SIM) mode. The solvent delay was 9.10 min. 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:

$$(\text{Mean Measured Concentration} - \text{Theoretical Concentration})/\text{Theoretical Concentration}] \times 100\%$$

Results

The retention times of flunitrazepam, 7-aminoflunitrazepam, and D_5 diazepam were approximately 9.81, 9.43 and 9.30 min, respectively. The chromatograms were recorded over the time range of 9.10 to 10.50 min. The standard curves for flunitrazepam and 7-aminoflunitrazepam were linear over the range of concentrations analyzed (2.5 to 15 pg/mg hair for flunitrazepam, and 0.5 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 and 0.5 pg/mg for flunitrazepam and 7-aminoflunitrazepam, respectively. Both limits of quantitation were arbitrarily

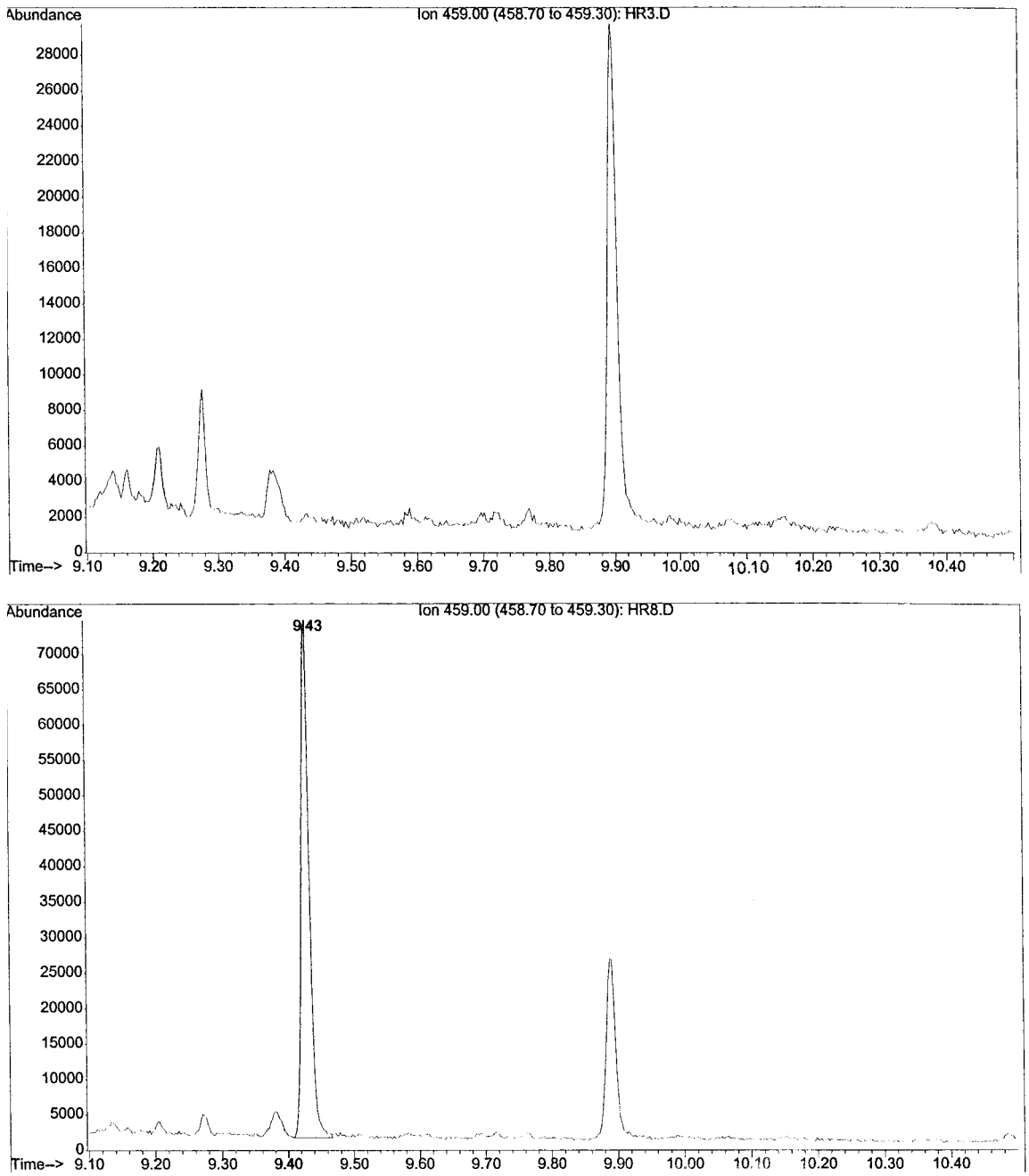

FIG. 1—*Selected ion chromatograms (*m/z *459) for subject #9 hair preparations before flunitrazepam administration (top) and three days after administration of the single 2 mg dose of the drug (7-aminoflunitrazepam conc. 7.4 pg/mg) (bottom).*

established to be the lowest concentrations on the corresponding standard curves.

Figure 1 shows representative selected ion chromatograms of 7-aminoflunitrazepam (*m/z* 459) for the hair preparations of subject #9 before flunitrazepam intake and three days after administration of a single 2 mg of flunitrazepam (7-aminoflunitrazepam concentration 7.4 pg/mg). Selected ion chromatograms of flunitrazepam (*m/z* 313) for the hair preparations of subject #9 before drug administration and three days after administration are shown in Fig. 2. The concentration of flunitrazepam was 2.3 pg/mg (below quantitation limit). Figure 3 presents typical selected ion chromatograms for the low control hair preparations for 7-aminoflunitrazepam (*m/z* 459, conc. 3 pg/mg) and flunitrazepam (*m/z* 313, conc. 4 pg/mg).

Tables 2 and 3 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 predrug hair specimens were negative for both flunitrazepam and 7-aminoflunitrazepam. The concentrations of fluni-

trazepam 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 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 4 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 h after flunitrazepam administration and remained in the hair throughout the entire 28-day study period (0.6–8.0 pg/mg) (Table 4). 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 (0.5–2.7 pg/mg) (Table 4). 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.

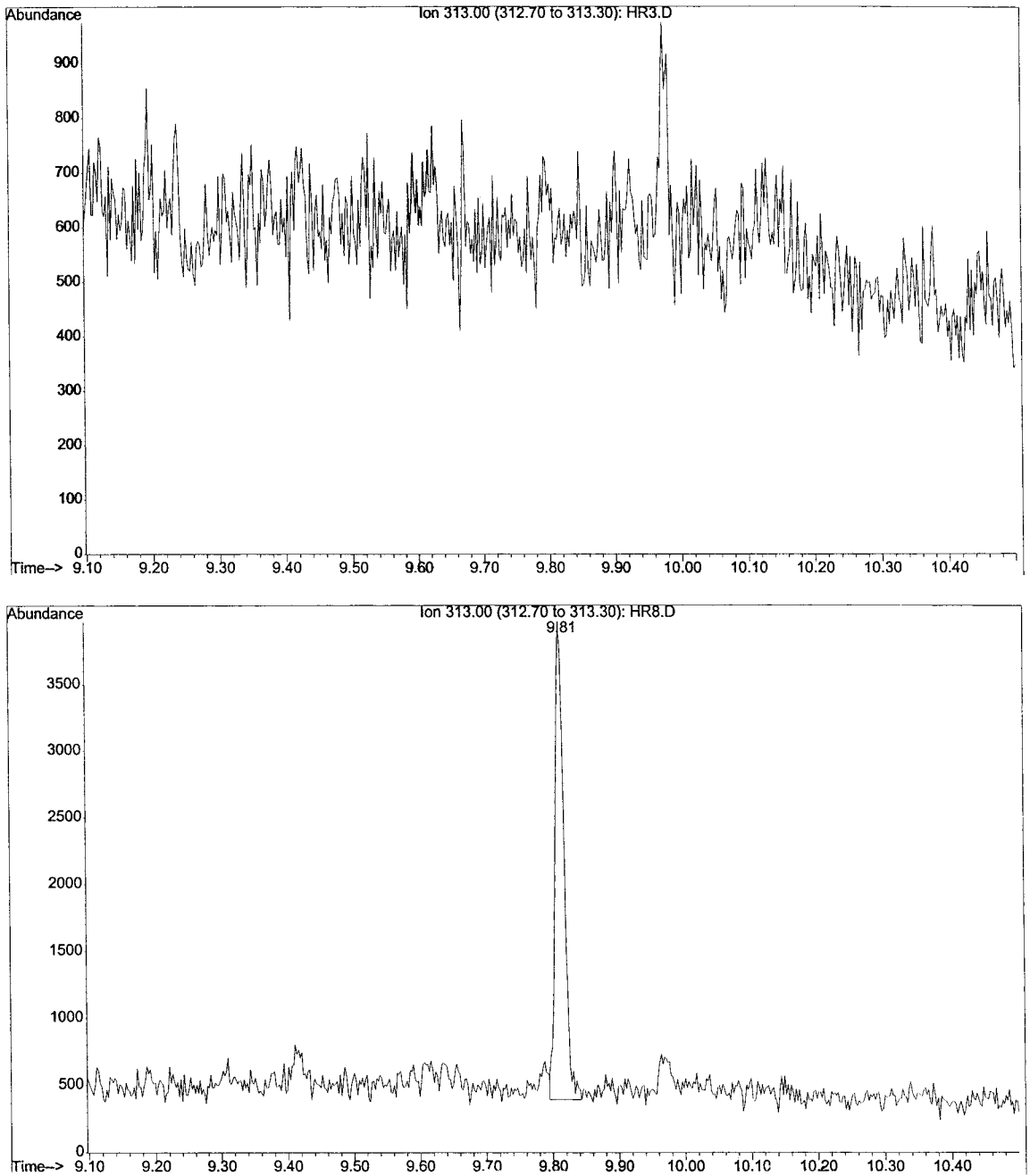

FIG. 2—*Selected ion chromatograms (*m/z *313) for subject #9 hair preparations before flunitrazepam administration (top) and three days after administration of the single 2 mg dose of the drug (flunitrazepam conc. 2.3 pg/mg) (bottom).*

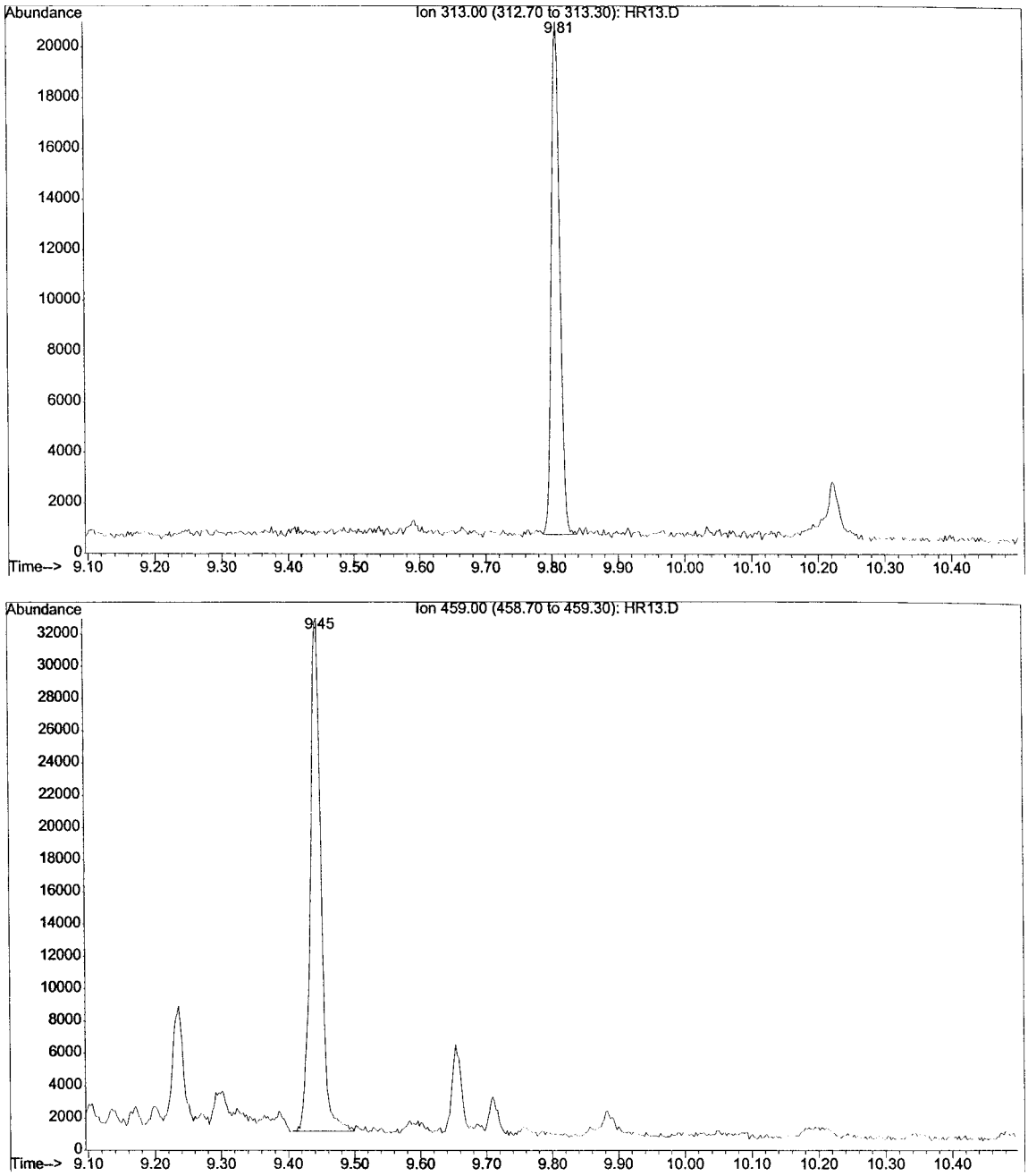

FIG. 3—*Selected ion chromatograms* m/z *313 (top) and* m/z *459 (bottom) for low control hair preparations (flunitrazepam conc. 4 pg/mg, 7-aminoflunitrazepam conc. 3 pg/mg).*

TABLE 2—*Accuracy and precision of flunitrazepam control hair preparations (pg/mg).*

Parameter	Low Control	High Control
Theoretical concentration	4	12
Intra-day variability	$N = 6$	$N = 6$
Mean measured concentration (±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 variability	$N = 15$	$N = 16$
Mean measured concentration (±S.D.)	3.4 (±0.4)	12.3 (±0.9)
% Coefficient of variation	11.8	7.3
% Relative accuracy	−15.0	2.5

TABLE 3—*Accuracy and precision of 7-aminoflunitrazepam control hair preparations (pg/mg).*

Parameter	Low Control	High Control
Theoretical concentration	3	15
Intra-day variability	$N = 6$	$N = 6$
Mean measured concentration (±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 variability	$N = 15$	$N = 16$
Mean measured concentration (±S.D.)	3.8 (±0.7)	15.5 (±2.1)
% Coefficient of variation	18.4	13.5
% Relative accuracy	26.6	3.3

TABLE 4—*7-Aminoflunitrazepam concentrations in subjects' hair (pg/mg).*

Subjects	Before Dosing	1 Day	3 Days	5 Days	14 Days	21 Days	28 Days
1	ND	2.0	5.8	—*	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†	0.4†	ND
9	ND	2.2	7.4	—*	5.0	6.4	8.0
10	ND	ND	ND	ND	0.6	0.3†	1.4

* Missing sample.
† Below quantitation limit.
ND: not detected.

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.

Discussion

During the last decade there has been an increase in the number of reports of drug-facilitated sexual assaults (1–5). In the early 1990s flunitrazepam, among other substances, was frequently mentioned as a "date-rape" drug (1,2,10) and for that reason has been banned in the United States. Flunitrazepam is also often abused by teens and young adults because of euphoric and "drunken-like" effect (10,11,22).

The presence of flunitrazepam and 7-aminoflunitrazepam in hair in relatively high quantities has been well documented (16–19,21,23). In our study, the concentration of 7-aminoflunitrazepam detected in the hair of each volunteer was greater than the concentration of flunitrazepam detected on each day. This can be explained by the fact that 7-aminoflunitrazepam is a more basic compound and will bind better to melanin than its parent drug, flunitrazepam. Cirimele et al. (17) found 26 hair specimens collected from corpses positive for 7-aminoflunitrazepam, flunitrazepam or both. The same author described the determination of chronic flunitrazepam abuse by analysis of a 10-cm hair strand divided into 3-cm segments collected from a chronic drug user (18). In another study (21), concentrations of 7-aminoflunitrazepam in two postmortem hair samples were found to be higher than corresponding flunitrazepam levels. Recently Negrusz et al. (24) discovered that the concentrations of 7-aminoclonazepam in hair samples collected from people chronically treated with clonazepam were much higher than those of clonazepam. In our study, we employed solid phase extraction and a sensitive NCI-GC-MS to simultaneously detect flunitrazepam and 7-aminoflunitrazepam in the hair of ten volunteer subjects who were administered a single 2 mg dose of Rohypnol®. The limit of detection for our method was 0.5 pg/mg for flunitrazepam and 0.2 pg/mg for 7-aminoflunitrazepam, and the limits of quantitation 2.5 and 0.5 pg/mg for flunitrazepam and 7-aminoflunitrazepam, respectively, which is much more sensitive than previous GC-MS methodology, making it a more suitable technique for forensic testing. Single dose administration of flunitrazepam, as in the case of a drug-facilitated sexual assault, results in hair-drug concentrations much lower than those detected in previous studies involving chronic flunitrazepam users (16–19). Similarly, in three separate studies by Cirimele et al. (16–18) and one study by Yegles et al. (23), the limits of detection for flunitrazepam ranged from 15 to 30 pg/mg and from 3 to 20 pg/mg for 7-aminoflunitrazepam. Previous work by Negrusz et al. (21) suggested that NCI-GC-MS is sensitive enough to detect quantities of 7-aminoflunitrazepam and flunitrazepam which would be found in hair after single dose administration. This is the first such study in which the amount of flunitrazepam administered was regulated in an attempt to determine whether this technique is sensitive enough to detect a single dose. In our study D_5 diazepam was chosen as an internal standard over deuterated analogs of 7-aminoflunitrazepam and flunitrazepam, since at such low concentrations D_7 7-aminoflunitrazepam and D_7 flunitrazepam may be contaminated with minute quantities of unlabeled compounds leading to false positive results.

Hair is assumed to grow at a constant rate. The position of drugs along the hair shaft should, in theory, be correlated with the time the drug was present in the bloodstream (25). As the hair grows, the position of the affixed drug should move away from the scalp at the same rate as the growth of the hair (26). Thus, it has been suggested that the position of the drug in the hair shaft could be indicative to the length of time since the person was exposed to the drugs. Nakahara et al. (27) and Cone (28) were able to demonstrate that the presence of drug along the hair shaft does correspond to the time of drug ingestion. From the data collected in our experiment, we were unable to show that hair analysis can be used to determine the exact timing of a drug-facilitated sexual assault. Based on the fluctuations in the 7-aminoflunitrazepam concentrations that we observed, even in this controlled situation, the exact date of administration would be difficult to extrapolate. In the case of a drug-facilitated sexual assault, the dose of drug ingested by the victim would not be known, therefore making this task impossible.

In our study, the hair collected from all ten subjects before Rohypnol® administration was negative, therefore eliminating the possibility of prior use of flunitrazepam by the volunteers. This finding led us to look at only the first segment of the hair shaft (1.5 cm from the root) based on the belief that drugs are incorporated into the hair primarily through the hair shaft via diffusion

from blood to the actively growing follicle (25). To determine if the timing of a sexual assault could be narrowed down to a relatively accurate time frame, we decided to analyze two additional hair segments (3–6 cm from the root and 6–9 cm from the root) from hair obtained on two collection days. Segments 2 and 3 were taken from hair collected on day 1 and day 14 from subject #9. Assuming that hair grows at a constant rate of 1.0 ± 0.3 cm/month, drug found in any other segment(s) would be most likely due to external contamination or via secretions of the apocrine and sebacious glands. In the two additional hair segments we analyzed, no flunitrazepam was detected in segment 2 or 3 on either day and only traces of 7-aminoflunitrazepam (below quantitation limit) were detected in the second segments from both day 1 and day 14. These findings indicate that the drug concentration does decrease down the length of the hair shaft away from the root which may help investigators narrow in on the time of flunitrazepam ingestion.

Another question surrounding the use of hair testing concerns the correlation between the dose of drug administered and the amount of drug in hair. In the previously mentioned studies, Nakahara et al. (27) found a good correlation between the concentration of methoxyamphetamine and the concentration of drug in the hair of five subjects. Likewise, Cone et al. found a positive correlation between the dose of opiates and the resulting concentration in beard hair (28). In our study, we were not able to determine any correlation between the amount of Rohypnol® administered and amount of drug detected in the hair of the subjects. The amount of flunitrazepam and 7-aminoflunitrazepam detected varied from subject to subject, despite the fact that the participants received the same dose of Rohypnol®. Our findings are similar to those by Puschel et al. (29) who analyzed hair obtained from carcinoma patients receiving therapeutic doses of morphine and found a poor correlation between the dose and the drug concentrations in hair. In fact, the highest concentrations were found in the hair of the subjects who received the lowest dose. These findings suggest that individual variability in bodily secretions or hair type, could partly explain the discrepancy in hair drug concentrations in subjects receiving the same dose.

In the case of a drug-facilitated sexual assault, the amount of drug ingested does not need to be determined in order to offer support to the prosecution's case. Any amount of flunitrazepam or 7-aminoflunitrazepam detected in the hair of the victim can be used as evidence of Rohypnol® ingestion. Whether the victim takes Rohypnol® knowingly or is unaware of its ingestion, sexual assault while under the influence of flunitrazepam is considered drug-facilitated sexual assault and can be prosecuted in the same manner.

External contamination and racial bias are often a cause of great concern when using hair for drug testing (30). Hair that is exposed to drugs from means outside of the individual's body, such as with drugs that are smoked or used in powder form, can result in positive drug tests. In these situations, the hair is often washed before analysis to ensure that external contamination is less of a factor. In our study, the hair from the volunteers was not washed because the presence of flunitrazepam in the individual's hair is indicative of exposure to the drug. Flunitrazepam is available in tablet form and any indication of flunitrazepam or 7-aminoflunitrazepam in the hair would most likely be due to ingestion alone. Similarly, racial bias concerns often raise questions about how to interpret the results of hair analysis by GC-MS (30,31). In one study, a more than ten-fold higher drug concentration was found in pigmented hair when compared to white (gray) hair (31). External contamination and racial bias do not play a factor in hair testing for drug-facilitated rape cases because the presence of flunitrazepam or 7-aminoflunitrazepam in the hair of a victim is evidence of ingestion of the drug and can be used as evidence to prosecute the suspect.

One major drawback concerning the use of hair testing as means of evidence in the case of drug-facilitated sexual assault is that there is evidence that flunitrazepam is being abused recreationally (11). There is little doubt that drug and/or alcohol use among young women constitute significant risk factors for sexual assault. From the standpoint of prosecuting the crime, the known recreational use of the same drugs that can be used to facilitate the rape of a woman without her knowledge can be an obstacle. Because of the amnesiac effects of these drugs, victims are often unable to provide good witness accounts of the assaults. The only evidence against the accused might then be identification as the semen depositor by DNA typing. In these cases, detection of one of the date-rape drugs or their metabolites in blood, urine, or hair taken from the victim could help support a contention of a drug-facilitated sexual assault.

The fact that drug-facilitated sexual assault cases require toxicological analysis in addition to the usual forensic biological examinations and DNA typing that are routine in most sexual assault cases is novel in U.S. forensic science labs. Therefore, specimens specifically for drug analysis will need to be collected which will require changes in sexual assault evidence kits and protocols. Recently, many sexual assault protocols have called for collecting a small bloodstain or a buccal swab, neither of which would serve the forensic toxicologist's purpose. In the past, head hair specimens had been collected from sexual assault victims for possible microscopical hair comparisons. Such a specimen may not be of use for toxicological analysis if it is collected too soon after the incident. Therefore, it is very important to determine how long after a single dose of drug, that hair samples could be collected from the victim to confirm the illegal drug's presence. It is clear that changes in protocols and kits will be required in the future. These changes may be made somewhat more difficult by the fact that many of the states have their own sexual assault evidence kits, and the procedures for changing the protocols and kits can be somewhat involved.

Based on the results that we obtained in this study, we recommend this instrumentation and method as a simple, relatively inexpensive and accurate procedure to be used for the analysis of hair in the case of a drug-facilitated sexual assault. Hair analysis can only be used in such cases if the sensitivity of the technique is enhanced to detect the levels of drug and/or drug metabolite that would be present in hair after a single dose. In our study, we were able to detect levels of flunitrazepam and 7-aminoflunitrazepam as low as 0.5 pg/mg after a single 2 mg dose of Rohypnol®. Flunitrazepam is a good example of a benzodiazepine that can be detected at low levels in hair because we are able to enhance its sensitivity through derivatization of its amino group. Research is still ongoing, but the detection of other benzodiazepines, such as diazepam, in hair after a single dose does not appear likely due to a lack of sensitivity. In a study done by Cirimele et al. (16), the limit of detection for diazepam was 11 pg/mg, which is significantly greater than any of the concentrations of flunitrazepam or 7-aminoflunitrazepam detected in our study after a single dose. The ability to detect flunitrazepam and 7-aminoflunitrazepam in hair in such small concentrations provides good support for the use of forensic hair testing in the case of a flunitrazepam-facilitated sexual assault.

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References

1. Smit KM. Drugs used in acquaintance rape. J Am Pharmac Assoc 1999 May/June;39(3):519–25.
2. LeBeau M, Andollo W, Lee Hearn W, Baselt R, Cone E, Finkle B, et al. Recommendations for toxicological investigations of drug-facilitated sexual assault. J Forensic Sci 1999;44(1):227–30.
3. ElSohly MA, Salamone SJ. Prevalence of drugs used in cases of alleged sexual assault. J Anal Toxicol 1999 May/June;23:141–6.
4. Rickert VI, Wiemann CM. Date rape among adolescent and young adults. J Pediatr Adolesc Gynecol 1998;11:167–75.
5. Kilpatrick DG, Acierno R, Resnick HS, Saunders BE, Best CL. A 2-year longitudinal analysis of the relationships between violent assault and substance use in women. J Consult Clin Psychol 1997;65(5):834–47.
6. *http://www.census.gov/prod/3/98pubs/98statab/cc98stab.htm*
7. Mattila MAK, Larni HM. Flunitrazepam: a review of its pharmacological properties and therapeutic use. Drugs 1980;20:353–74.
8. Bogusz MJ, Maier R-D, Krüger K-D, Früchtnicht W. Determination of flunitrazepam and its metabolites in blood by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J Chromatogr B 1998;713:361–9.
9. ElSohly MA, Feng S, Salamone SJ, Wu R. A sensitive GC-MS procedure for the analysis of flunitrazepam and its metabolites in urine. J Anal Toxicol 1997 Sep;21:335–40.
10. Simmons MM, Cupp MJ. Use and abuse of flunitrazepam. Ann Pharmacother 1998 Jan;32:117–9.
11. Rickert VI, Wiemann CM, Berenson AB. Prevalence, patterns, and correlates of voluntary flunitrazepam use. Pediatrics 1999 Jan;103(1):1–5.
12. ElSohly MA, Feng S, Salamone SJ, Brenneisen R. GC-MS determination of flunitrazepam and its major metabolite in whole blood and plasma. J Anal Toxicol 1999 Oct;23:486–9.
13. Song D, Zhang S, Kohlhof K. Quantitative determination of clonazepam in plasma by gas chromatography-negative ion chemical ionization mass spectrometry. J Chromatogr B 1996;686:199–204.
14. Fitzgerald RL, Rexin DA, Herold DA. Benzodiazepine analysis by negative chemical ionization gas chromatography/mass spectrometry. J Anal Toxicol 1993 Oct;17:342–7.
15. Kintz P, Cirimele V, Vaysette F, Mangin P. Hair analysis for nordiazepam and oxazepam by gas chromatography-negative-ion chemical ionization mass spectrometry. J Chromatogr 1996;677:241–4.
16. Cirimele V, Kintz P, Ludes B. Screening for forensically relevant benzodiazepines in human hair by gas chromatography-negative ion chemical ionization-mass spectrometry. J Chromatogr B 1997;700: 119–29.
17. Cirimele V, Kintz P, Staub C, Mangin P. Testing human hair for flunitrazepam and 7-aminoflunitrazepam by GC/MS-NCI. Forensic Sci Int 1997;84:189–200.
18. Cirimele V, Kintz P, Mangin P. Determination of chronic flunitrazepam abuse by hair analysis using GC-MS-NCI. J Anal Toxicol 1996;20: 596–8.
19. Kintz P, Tracqui A, Mangin P. Detection of drugs in human hair for clinical and forensic applications. Int J Leg Med 1992;105:1–4.
20. Negrusz A, Moore CM, Stockham TL, Poiser KR, Kern JL, Palaparthy R, et al. Elimination of 7-aminoflunitrazepam and flunitrazepam in urine after a single dose of Rohypnol®. J Forensic Sci 2000 Sep;45(5): 1031–40.
21. Negrusz A, Moore C, Deitermann D, Lewis D, Kaleciak K, Kronstrand R, et al. Highly sensitive micro-plate enzyme immunoassay screening and NCI-GC-MS confirmation of flunitrazepam and its major metabolite 7-aminoflunitrazepam in hair. J Anal Toxicol 1999 Oct;23:429–35.
22. Calhoun SR, Wesson DR, Galloway GP, Smith DE. Abuse of flunitrazepam (Rohypnol) and other benzodiazepines in Austin and South Texas. J Psychoactive Drugs 1996 April/June;28(2):183–9.
23. Yegles M, Mersch F, Wennig R. Detection of benzodiazepines and other psychotropic drugs in human hair by GC/MS. Forensic Sci Int 1997;84: 211–8.
24. Negrusz A, Moore CM, Kern JL, Janicak PG, Strong MJ, Levy NA. Quantitation of clonazepam and its major metabolite 7-aminoclonazepam in hair. J Anal Toxicol 2000;24(7):614–20.
25. Henderson GL. Mechanisms of drug incorporation into hair. Forensic Sci Int 1993;63:19–29.
26. Bost RO. Hair analysis—perspectives and limits of a proposed forensic method of proof: a review. Forensic Sci Int 1993;63:31–42.
27. Nakahara Y, Shimamine M, Takahahi K. Hair analysis for drugs of abuse. III. Movement and stability of methoxyphenamine (as a model compound for methamphetamine) along hair shaft with hair growth. J Anal Toxicol 1992;16:253–7.
28. Cone E. Testing human hair for drugs of abuse. I. Individual dose profiles of morphine and codeine in plasma, saliva, urine, and beard compared to drug-induced effects on pupils and behavior. J Anal Toxicol 1991;14:1–7.
29. Puschel K, Thomasch P, Arnold W. Opiate levels in hair. Forensic Sci Int 1983;21(2):181–6.
30. Sachs H, Kintz P. Testing for drugs in hair: critical review of chromatographic procedures since 1992. J Chromatogr B 1998;713:147–61.
31. Rothe M, Pragst F, Thor S, Hunger J. Effect of pigmentation on the drug deposition in hair of grey-haired subjects. Forensic Sci Int 1997;84: 53–60.

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