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Elimination of 7-Aminoflunitrazepam and Flunitrazepam in Urine After a Single Dose of Rohypnol®*

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ABSTRACT: The hypnotic benzodiazepine flunitrazepam (Rohypnol®) has been identified as the drug of choice for the purposes of "drugging" unsuspecting victims and raping them while they are under the influence of this substance. The objective of this paper was to study elimination of flunitrazepam and 7-aminoflunitrazepam in urine collected from ten healthy volunteers who received a single 2 mg oral dose of Rohypnol®, to determine how long after drug administration 7-aminoflunitrazepam can be detected. A highly sensitive NCI-GC-MS method for the simultaneous quantitation of flunitrazepam (LOQ 100 pg/mL) and 7-aminoflunitrazepam (LOQ 10 pg/mL) in urine was developed. All samples were screened for benzodiazepines using optimized micro-plate enzyme immunoassay. The highest concentrations of 7-aminoflunitrazepam (70–518 ng/mL) and flunitrazepam (0.7–2.8 ng/mL) in urine were observed 6 h after drug administration in nine subjects and after 24 h in one subject. In six subjects 7-aminoflunitrazepam was detected up to 14 days after flunitrazepam administration, in one subject up to 21 days and in three subjects up to 28 days. In urine samples collected from six volunteers, flunitrazepam was detected three days after Rohypnol® intake, in three subjects 24 h, and in one subject 5 days later. Benzodiazepine micro-plate enzyme immunoassay kit allowed the detection of flunitrazepam and metabolities 5 to 21 days after drug administration.

KEYWORDS: forensic science, forensic toxicology, drug-facilitated sexual assault, 7-aminoflunitrazepam, flunitrazepam, drug elimination, solid-phase extraction, microplate immunoassay, NCI-Gas Chromatography-Mass Spectrometry

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 (1). Its hypnotic effect predominates over the sedative, anxiolytic and muscle-relaxing effects of other compounds from the same pharmacological group. Flunitrazepam (FN) is available in oral tablets and in injectable form (2) in about 80 countries around the world. FN has much stronger affinity for the GABA receptor than that of diazepam (Valium®). In fact, it is 10 times as potent as diazepam (1,2). FN is readily (80–90%) absorbed through the gastrointestinal tract and metabolized almost completely by the liver. It includes reduction to 7-aminoflunitrazepam (7-AFN) and then to the N-glucuronide, demethylation to the N-demethyl metabolite, hydrolysis to the 3-OH metabolite and then to the O-glucuronide (1,3,4). Approximately 90% of its metabolites are excreted through the urine and 10% in the feces (1,4). Deaths involving FN have occurred in conjunction with other CNS depressants, such as ethanol, but also due to overdose of FN alone (5).

Rohypnol®'s first seizure in the US took place in 1989, and reports of the misuse of the drug have increased since then (2,6). FN is often used to enhance the effects of heroin, alcohol, or marijuana (2). Voluntary FN use in combination with alcohol and other illicit drugs in Texas among sexually active adolescent and young adult women 14–26 years of age was reported by Rickert et al. (7). In a recent paper, Raymon et al. reported significant involvement of FN (among other benzodiazepines) in driving under the influence cases in South Florida between 1995 and 1998 (8). Rohypnol® tablets are smuggled to the US (mainly from Mexico) and sold with the following street names: roofies, rophies, roopies, rib, rope, pappas, peanuts, pastas, forget pills, ro-shays, roaches, roche 2 (2). In the early 1990s FN 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 (2,6,9), and classified as so called "date-rape" drug. As of 1996, the prescription, sale, and importation of FN into the US has been banned. With specific regard to the use of FN in rape cases, 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. The majority of rape victims do not report the incident until several days after the event. The reason for this is a combination of emotions, including embarrassment, fear, rejection, denial, mistrust in authorities and often amnesia caused by the drug. It is therefore very important to determine how long after drug-facilitated sexual assault urine samples could be collected from the victim to confirm illegal drug use and to successfully prosecute the perpetrator. In whole blood FN has been de-

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tected only up to 4 h after single 2 mg dose of the drug (10). In the same study, 7-AFN was detected in higher concentrations in plasma than in blood for the entire 12-h period of specimen collection. The limit of detection (LOD) for FN in blood was 5 ng/mL. ElSohly et al. presented a EI-GC-MS method for detection of FN and its major metabolites in urine collected from volunteers who received single 1, 2, and 4 mg dose of the drug (4). The LOD for corresponding benzophenones was 1 ng/mL. The described GC-MS method allowed the detection of FN metabolites in urine 72 h after a single dose of the drug. Similar urine excretion patterns after single 1 and 4 mg oral dose of FN using two immunoassays with and without β-glucuronidase treatment followed by GC-MS were described by Salamone et al. (11).

The first objective of this paper was to develop a sensitive, precise, accurate, easily available and relatively inexpensive negative ion chemical ionization gas chromatography-mass spectrometry (NCI-GC-MS) method for detection of 7-AFN and FN in human urine with potential application to alleged drug-facilitated rape victims. The second goal was to test how long after administration of a single dose of Rohypnol®, FN and its major metabolite 7-AFN can be detected in urine using both NCI-GC-MS and a commercially available, highly sensitive micro-plate enzyme immunoassay.

Methods

Subjects and Specimens

Ten volunteers (eight women and two 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® (FN) 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. Urine samples were collected according to the following schedule: one sample prior to drug administration, and 6 h, 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 and by the Institutional Review Board of the University of Illinois at Chicago.

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-AFN 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-AFN and FN 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 h at 37°C. 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 h at 37°C. 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°C 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 min. The plate was emptied and washed with deionized water (6 × 300 μL). Substrate reagent was added (100 μL). The plate was again incubated in the dark, at room temperature, for 30 min. 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-AFN 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 × 250 μm × 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

FN (1 mg/mL in methanol), 7-AFN (100 μg/mL in acetonitrile), and the deuterated internal standards D_7 FN (100 μg/mL in methanol) and D_7 7-AFN (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), iso-

propanol (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 FN (1 mg/mL in methanol) standard stock solution was diluted to 100, 10, 1, and 0.2 μg/mL. The 7-AFN (100 μg/mL in acetonitrile) standard stock solution was diluted to 10 μg/mL, 0.1 μg/mL, and 10 ng/mL. The D_7FN (100 μg/mL in methanol) and D_7 7-AFN (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 FN and 7-AFN using negative urine spiked with solutions of both drugs. The concentrations of the FN in standard urine preparations were as follows: 100, 300, 500, 700, 1000, 1500, and 2000 pg/mL of urine. The concentrations of 7-AFN 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 FN (200 pg/mL) and 7-AFN (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 FN (1800 pg/mL) and 7-AFN (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 FN and 5 μL of 100 pg/μL of D_7 7-AFN 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 h at 37°C, 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°C. The samples were derivatized using 50 μL of HFBA at 60°C for 30 min in the sealed vials. After incubation, the derivatizing agent was evaporated in the vacuum oven (60°C) 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°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 min. The following ions were monitored and used for quantitation: for FN *m/z* 313 and 297, D_7 FN *m/z* 320, for 7-AFN *m/z* 459 and 441, and for D_7 7-AFN *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 FN is particularly sensitive to active sites.

Precision and Accuracy

Quantitation of FN and 7-AFN 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 FN to the peak area of the internal standard, D_7 FN. A separate seven-point standard curve was also prepared for 7-AFN with D_7 7-AFN 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 (1800 pg/mL, 800 pg/mL) for FN and 7-AFN, 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] × 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 FN 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-AFN, FN 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 h after administration of a single dose of Rohypnol®. The absorbance values, which ranged from 1.25 to 1.79 (area between two lines) represent urine samples negative for FN 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 FN-related compounds in unextracted urine and urine hydrolyzed without ex-

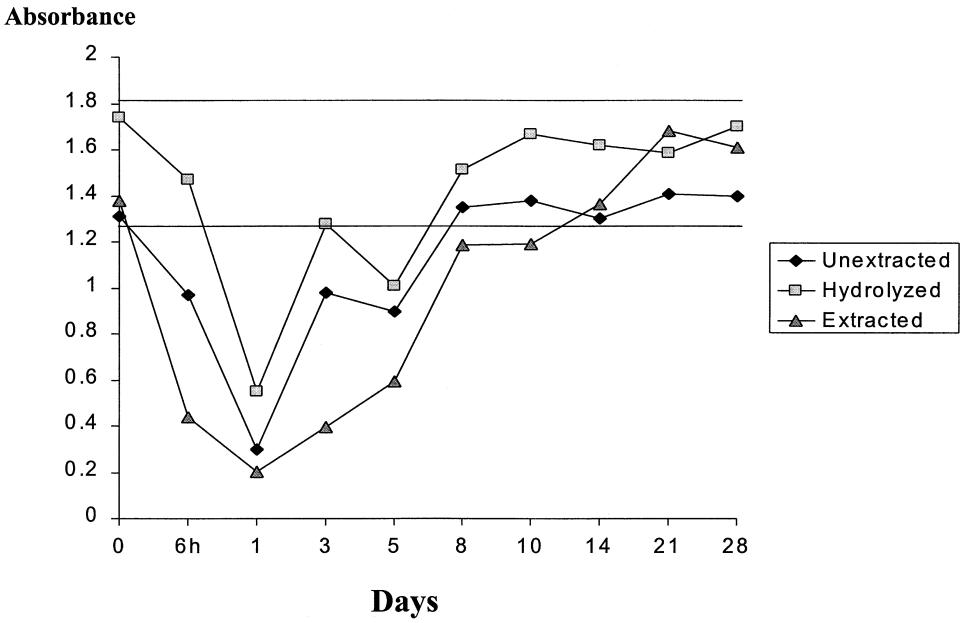

FIG. 1—*Micro-plate enzyme immunoassay analysis of urine samples collected from the same subject using three different sample preparation steps: no pre-treatment, enzymatic hydrolysis, enzymatic hydrolysis followed by solid-phase extraction.*

traction was possible only during the first 5 days after drug administration.

Chemical Ionization Gas Chromatography-Mass Spectrometry

An undiluted urine extract from volunteer #1 (6 h after Rohypnol® administration) is presented in Figs. 2 and 3. The concentrations of 7-AFN and FN were 232 ng/mL (Fig. 2) and 2.8 ng/mL (Fig. 3), respectively. Figure 4 shows the typical ion chromatogram of the extract from a 14-day urine collection: the concentration of 7-AFN was 120 pg/mL. The retention times of 7-AFN, D_7 7-AFN, FN and D_7 FN were approximately 9.53, 9.52, 9.94, and 9.92, min, respectively. All chromatograms were recorded over the time range 9.00 to 11.00 min. Standard curves for FN and 7-AFN were linear over the range of drugs assayed (10 pg/mL to 1000 pg/mL for 7-AFN and 100 pg/mL to 2000 pg/mL for FN) and had correlation coefficients 0.977 and 1.000, respectively. The limit of quantitation for 7-AFN was 10 pg/mL and for FN 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 7-AFN was 3 pg/mL and for FN 50 pg/mL, which was the lowest concentrations of drugs at which the signal-to-noise ratio was 3:1.

Tables 1 and 2 present the accuracy and precision of the FN and 7-AFN 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 (1800 pg/mL, 800 pg/mL) for FN and 7-AFN, respectively. Inter-day variability was ascertained over a period of six weeks.

In three subjects (volunteers #2, #4, and #8), 7-AFN was detected throughout the entire 28-day study period (detected concentrations were 16.5 to 22.1 pg/mL). Figure 5 presents the elimination of 7-AFN in urine in all subjects. FN 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 (Fig. 6). Urine samples collected from two volunteers were positive for FN only during the first 24 h. FN concentrations in urine never exceeded 3 ng/mL. Figures 7 and 8 present the results for analysis of urine samples collected from ten subjects at 6-h and 14-day time points, respectively. The highest concentration of 7-AFN in 6-h urine collection was 518 ng/mL and the lowest 70 ng/mL. In 14-day collection the highest 7-AFN urine concentration was 500 pg/mL (subject #3) and the lowest 40 pg/mL (subject #10). In nine subjects the maximum concentration of 7-AFN in urine was observed 6 h after drug administration and in one subject (volunteer #9) at 24-h time point. Figure 9 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.

Discussion

During the last decade, there has been an increase in the number of reports of drug-facilitated sexual assault (2,9,12–14). In the early 1990s FN, among other substances, was frequently mentioned as a "date-rape" drug (2,9,13,15) and for that reason has been banned in the United States. FN is also often abused by teens and young adults because of euphoric and "drunken-like" effect (6,7,15). In 1999, Rickert et al. (7) presented results of the cross-sectional survey on 904 sexually active women 14 to 26 years of age. Of the 904 women, 5.9% admitted using FN voluntarily one or more times, 7.5% of those who used FN reported using it 10 to 19 times, and 11% reported using it 20 or more times. In 1995 and 1996 FN was also the most frequent benzodiazepine in Miami-Dade County DUI-related urine samples often used alone or in combination with marijuana and cocaine (8). In the

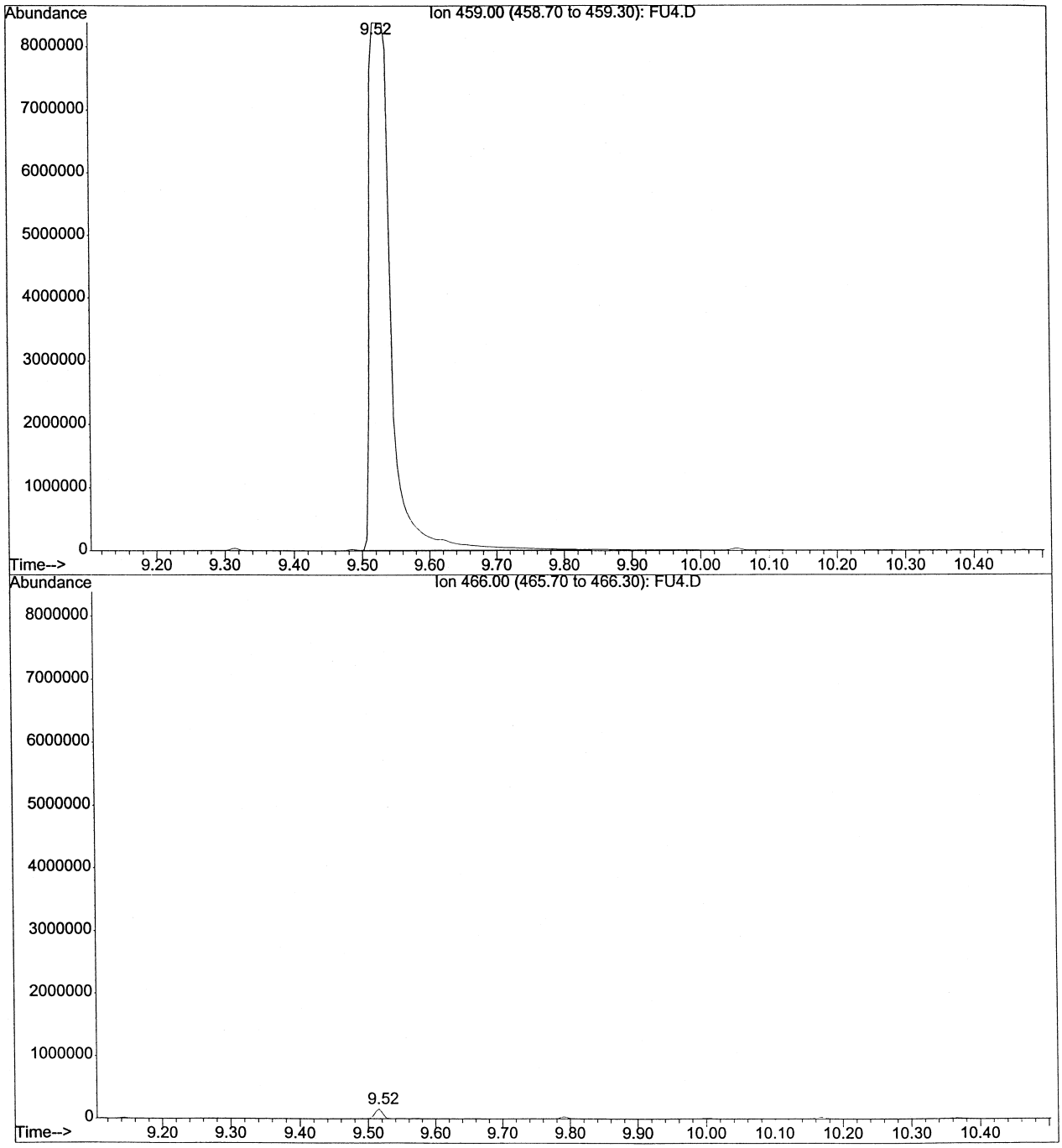

FIG. 2—*Selected ion chromatograms (*m/z *459 and 466) of the urine extract from volunteer #1 6 h after administration of a single 2 mg dose of Rohypnol® (7-AFN concentration 232 ng/mL).*

middle of the 1990s, law enforcement agencies and rape crisis centers recommended more sophisticated testing of specimens collected from alleged rape victims and a nationwide urine testing program for drugs used to commit this crime was developed (12). One of the most important issues, which needs to be taken under consideration in drug-facilitated sexual assault investigation is the sensitivity of both screening and confirmatory technique since some of the compounds, e.g., benzodiazepines, are typically used in a single low dose (9). Beck et al. (16) compared three commercially available techniques (EMIT, FPIA, and Online) for urine screening for benzodiazepines, including FN and 7-AFN. In their study, a much better positive rate was achieved when enzymatic hydrolysis (β-glucuronidase) was included. In another study, Salamone et al. (11) was studying excretion patterns of FN and its metabolites in urine after a single dose (1,2, and 4 mg) using Abuscreen OnTrak and OnLine immunoassays and GC-MS. The last positive result after 4 mg dose ranged from 18 to 60 h after drug administration. Enzymatic hydrolysis also increased On-

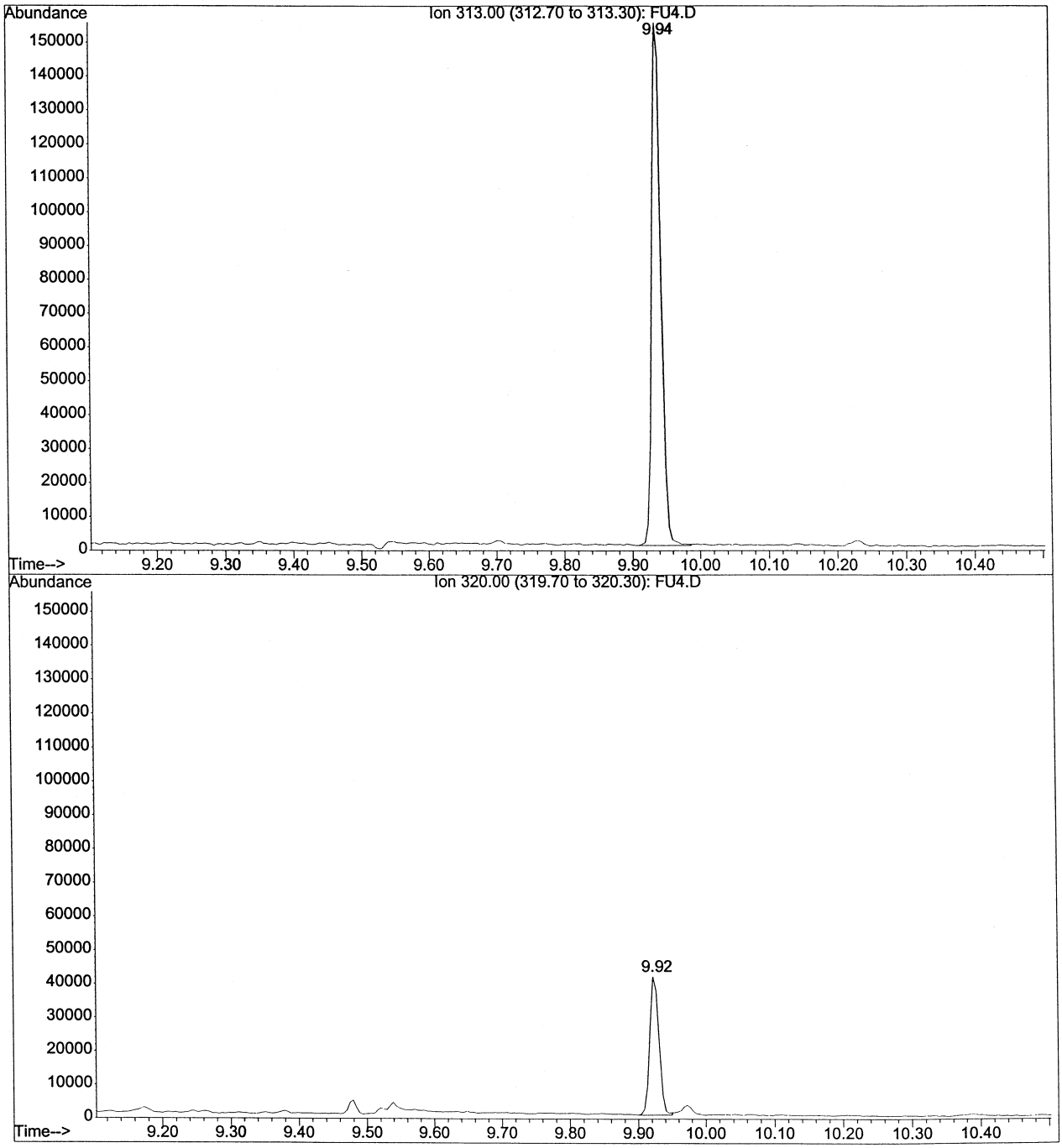

FIG. 3—*Selected ion chromatograms (*m/z *313 and 320) of the urine extract from volunteer #1 6 h after administration of a single 2 mg dose of Rohypnol® (FN concentration 2.8 ng/mL).*

Line values 20–60% but did not increase the detection time. In our study enzymatic hydrolysis with β-glucuronidase followed by solid-phase extraction was employed. Using micro-plate enzyme immunoassay we were able to detect FN and its metabolites in subjects' urine up to 21 days in one volunteer, 10 days in two cases, 8 days in 4 subjects and 5 days in three subjects after a single oral dose of Rohypnol®. The detectable levels of FN and its metabolites were observed in urine five days after drug intake regardless of urine treatment used (Fig. 1). We, therefore, recommend commercially available micro-plate enzyme immunoassay in sexual assault investigation when FN or other benzodiazepine use is expected.

Negative chemical ionization GC-MS improves the sensitivity and specificity for quantification of benzodiazepines in various biological specimens significantly exceeding those for traditional electron ionization GC-MS (18–20). In our study the presence of FN and its major metabolite 7-AFN in urine after a single 2 mg dose of Rohypnol® was confirmed using highly sensitive NCI-GC-

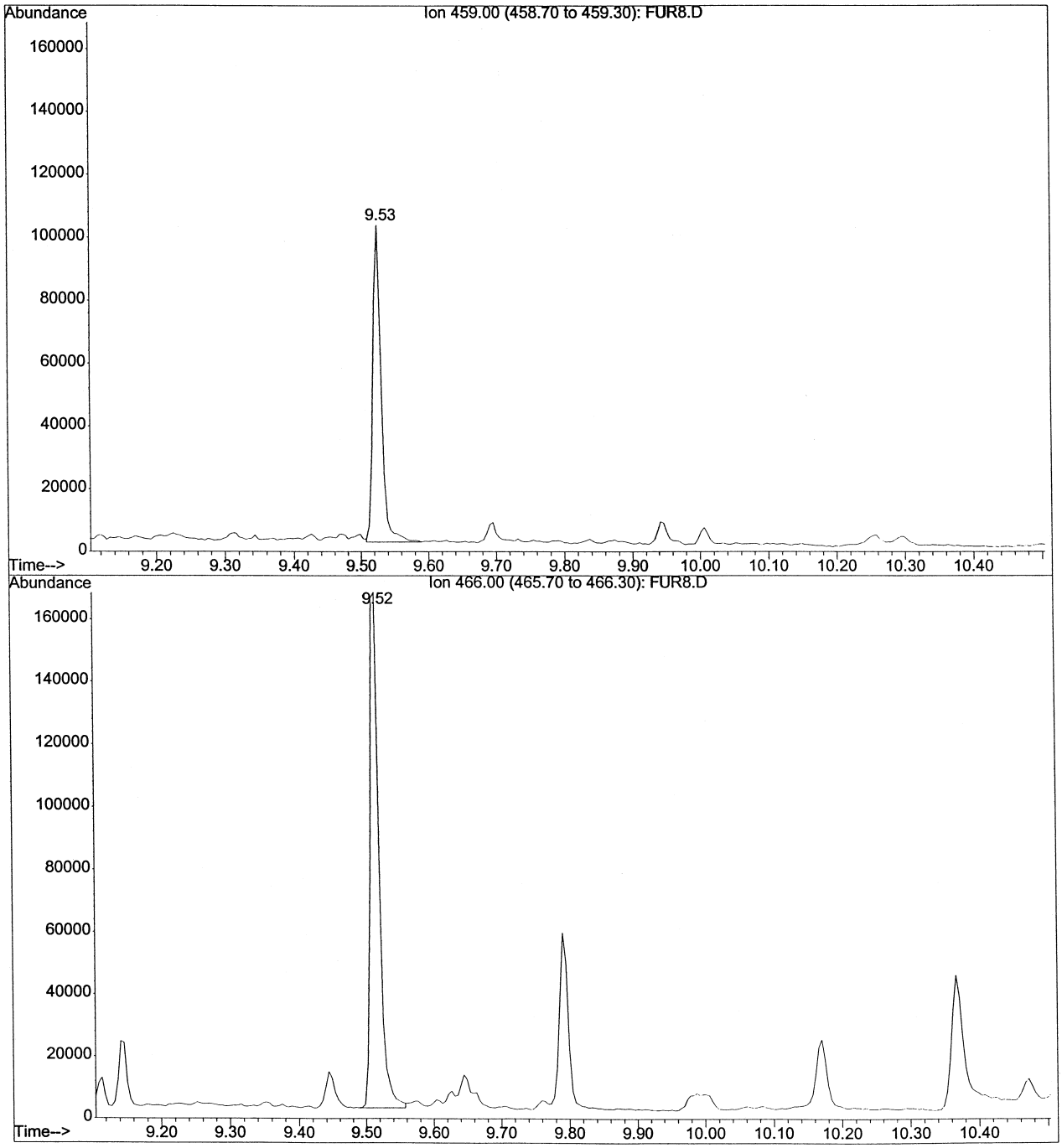

*FIG. 4—Selected ion chromatograms (*m/z *459 and 466) of the urine extract from volunteer #1 14 days after administration of a single 2 mg dose of Rohypnol® (7-AFN concentration 120 pg/mL).*

TABLE 1—*Accuracy and precision of FN 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)	1883.61 (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)	1740.76 (280.32)
% Coefficient of Variation	14.54	16.10
% Relative Accuracy	6.56	−3.29

TABLE 2—*Accuracy and precision of 7-AFN 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

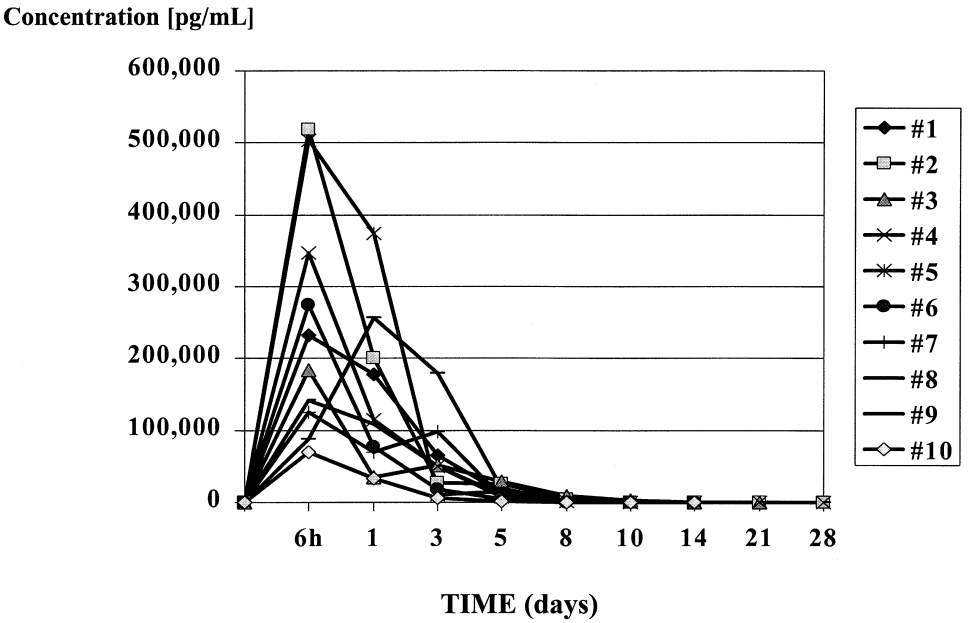

FIG. 5—*Elimination of 7-AFN in urine in all subjects after administration of a single 2 mg dose of Rohypnol®.*

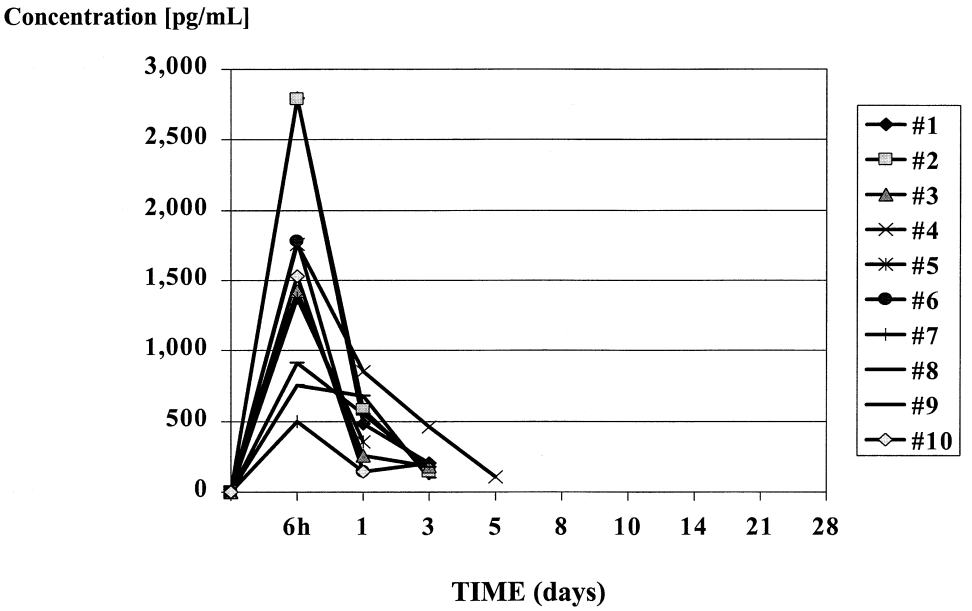

FIG. 6—*Elimination of FN in urine in all subjects after administration of a single 2 mg dose of Rohypnol®.*

MS. A similar analytical procedure for the quantitation of FN and 7-AFN in hair with NCI-GC-MS application was previously described by Negrusz et al. (17). The quantitation limit for 7-AFN was 0.5 pg/mg of hair. ElSohly et al. published EI-GC-MS methodology for detection of FN and its major metabolites in urine (4) and in whole blood and plasma (10) after hydrolysis to benzophenones, solvent extraction and derivatization. The limits of detection for these specimens ranged from 1 ng/mL to 5 ng/mL and the drug was determined in urine 72 h after a single 1 or 2 mg dose of FN and in blood and plasma (7-AFN) for the entire period of sample collection (12 h). In our study we were able to achieve a quantitation level of 10 pg/mL for 7-AFN (after derivatization with HFBA) and 100 pg/mL for FN. Urine samples were hydrolyzed with β-glucuronidase and the analytes were extracted using solid-phase mixed mode columns. All precision and accuracy values were within acceptable limits (Tables 1 and 2). The application of NCI-

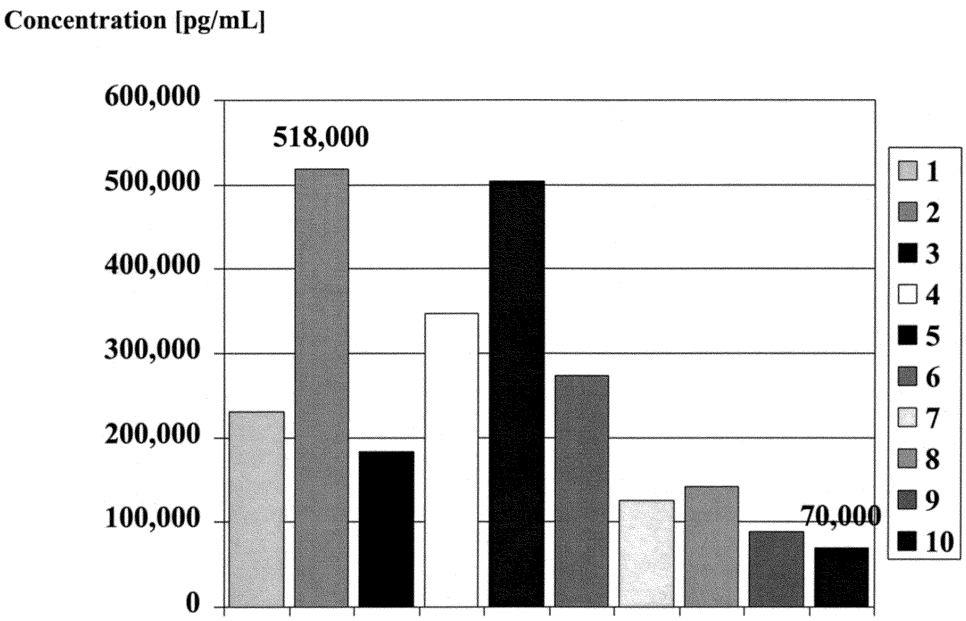

FIG. 7—*7-AFN concentrations in 6 h urine collection from all volunteers.*

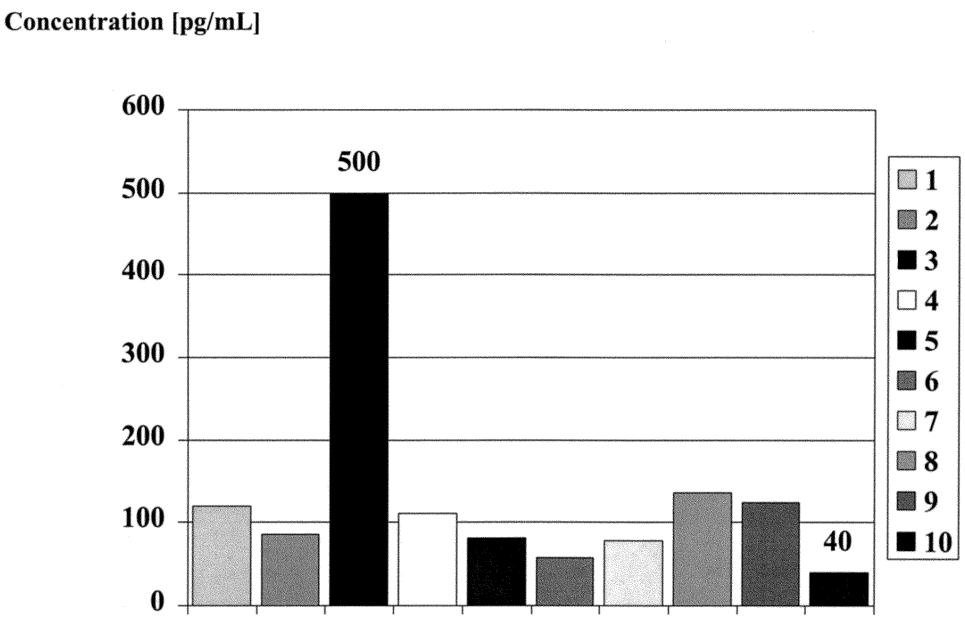

FIG. 8—*7-AFN concentrations in 14-day urine collection from all study subjects.*

GC-MS allowed us to detect and quantify 7-AFN in urine samples (using two ions) after a single 2 mg dose of Rohypnol® up to 28 days in three out of ten subjects, 21 days in one subject and 14 days in the remaining six subjects. Unchanged FN was present in urine samples up to 5 days in only one subject, 3 days in seven subjects and 24 h in two subjects. Our controlled clinical study on elimination of FN and its predominant urine metabolite 7-AFN clearly indicates, that the urine specimens could be collected from the rape victims for at least 14 days after the alleged incident to successfully prosecute the perpetrator. Relatively inexpensive and easily available negative chemical ionization GC-MS benchtop instrumentation provides 1000 fold improvement in sensitivity over EI-GC-MS and greatly extends the period during which Rohypnol® can be detected, an important advantage in cases of drug-facilitated sexual assault where there can be substantial delays in reporting the crime and because of the relatively low dose that is usually administered.

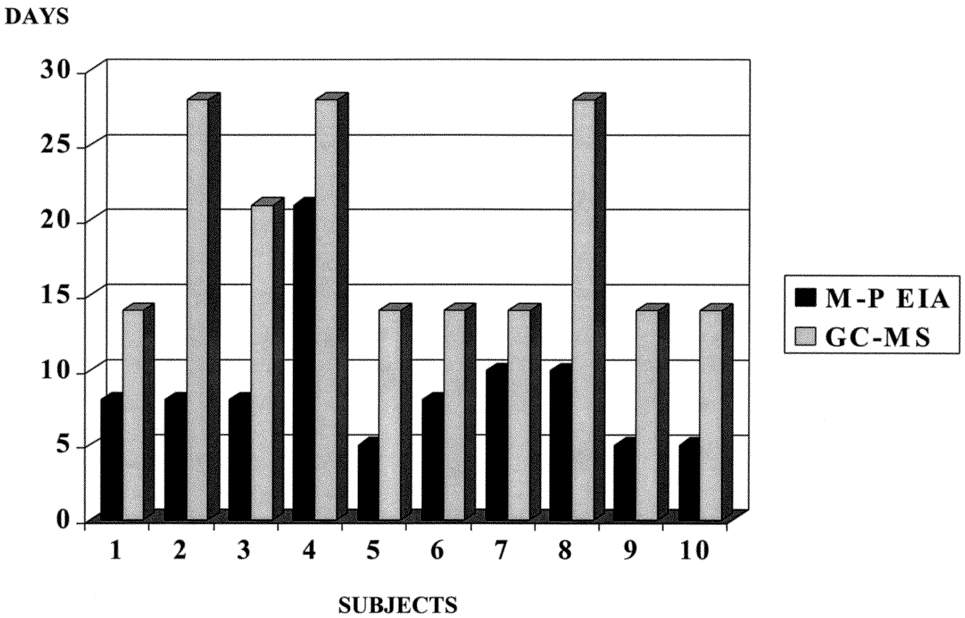

FIG. 9—*Results of urine analysis by NCI-GC-MS vs. micro-plate enzyme immunoassay after enzymatic hydrolysis and solid-phase extraction.*

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