

Hair analysis of drugs involved in drug-facilitated sexual assault and detection of zolpidem in a suspected case

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Abstract In drug-facilitated crimes, victims are subjected to nonconsensual acts while they are incapacitated by the effects of a drug. A specific LC-MS/MS protocol for determining benzodiazepines and hypnotics at low concentration in hair specimens was developed and validated in order to target the allegedly administered drugs on a chronological basis. In the case hereby reported, a 26-year-old woman claimed to have been sexually assaulted after being administered an allegedly drugged coffee, but toxicological analysis of urine and blood provided no evidence of any drug intake. Subsequently, a second woman accused the same man of sexual abuse. Hence, the suspect was prosecuted. Specimens were collected from

four subjects (two alleged victims, the suspect and his wife) and segmental hair analysis was performed. The results revealed that zolpidem was present at low picogram per milligram concentration in three out of eleven segments of hair specimen obtained from the first of the alleged victims, offering plain evidence of single or sporadic exposure, whereas the agent was detected in the high picogram per milligram range in the hair collected from suspect's wife, coherently with therapeutic administration. The presence of interfering signals typical of the keratin-containing matrix was found and possible hair degradation by cosmetic treatments was investigated by electron microscopy, so as to obtain a judicious interpretation of the analytical findings.

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Introduction

The determination of drugs in hair samples is recognized as a fundamental tool for toxicologists. Once incorporated into growing hair, most drugs can be detected over a significant period, usually as long as the hair is not cut. Therefore, hair analysis has been used for decades to indicate long-term assumption or drug addiction. In most drug-facilitated crimes, the victims are subjected to violence or nonconsensual acts while they are incapacitated by the effects of a drug, often unawares ingested. The drugs utilized for criminal purposes include benzodiazepines, hypnotics, sedatives and anesthetics, drugs of abuse (such as cannabis, ecstasy, LSD, or heroin), miscellaneous drugs (e.g., scopolamine) and, most fre-

quently, ethanol [1]. Many potential “rape drugs” were also identified and comprehensively listed [2]. The ideal substance for perpetrating a crime is the one that is readily available, easy to administer, able to rapidly impair consciousness, and produce anterograde amnesia. Many of the drugs used in drug-facilitated sexual assaults (DFSA) are fast acting and strong central nervous system depressants, which may trigger multiple pharmacological effects such as relaxation, euphoria, decreased inhibition threshold, amnesia, impaired perceptions, difficulty in maintaining equilibrium, impaired speech, drowsiness, loss of motion, vomiting, incontinence, unconsciousness, and occasionally death [3]. Since the depressant effects of most of these drugs are generally similar, it is highly unlikely that the specific drug used in a sexual assault could be determined only by symptoms.

It is well known that sexual assaults are significantly under- and lately reported. Evidently, any delay in reporting the assault implies that specimens are not timely collected, hence rendering the effort more difficult for toxicologists to identify the incapacitating agent used [4]. Hair is the most helpful specimen in all situations where natural metabolic processes have eliminated the drug from biological fluids (typically, urine and blood), in cases of late crime report [5–7]. Consequently, toxicology laboratories are frequently asked to collect and analyze hair from the victims in order to reveal the allegedly administered drugs. Furthermore, segmental analysis generally provides chronological information. Assuming that the growth rate of human scalp hair is approximately 1 cm/month [8], administration of a single dose can be confirmed by the detection of the drug in the correspondent segment, with no presence in the preceding and subsequent segments. The expected concentration in hair is generally in the low picogram/milligram range for most drugs. Therefore, the use of highly sensitive instrumental techniques, such as gas or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), is mandatory in the investigation of these drug-facilitated crimes [9–12].

Case reports

In the case presently described, two women were involved. Both reported to the police that they had been sexually abused after accepting a drink from the same man, at different times. Thereafter, they allegedly lost memory and consciousness for several hours. The first woman (Caucasian, 26-year-old) claimed to have been sexually assaulted while at work. She remembered that her employer offered her a coffee, which had a bitter taste. Initially, she could not recollect what happened thereafter, but, when she recovered full consciousness, she gradually recalled that the man submitted her to

personal palpations. Three days later, she went to the hospital, where urine and blood samples were collected. After being informed about this case from an acquaintance they had in common, a second woman (African, 41-year-old) reported to the police an earlier but analogue episode, in which the same man allegedly came to her apartment offering coffee and orange juice. After drinking, she lost consciousness and woke up the next morning observing vaginal bleeding and anomalies in the clothes she wore. After these two reports, the man was prosecuted. For the purposes of the investigation, the two women were asked to give hair samples 5 and 17 months, respectively, after the alleged abuse. Hair samples were also collected from the accused, as well as his wife in order to verify if they were habitual consumers of any psychoactive drug, in particular Halcion® (triazolam) and Zolpidem Teva® (zolpidem), whose blister packs were seized by the police at both their domicile and workplace.

All analyses were carried out using LC-MS/MS procedures. Hair samples were also investigated by scanning electron microscopy in order to evaluate the condition and preservation of the hair cuticle several months after the reported episodes.

Experimental

Chemicals and reagents

Flunitrazepam, diazepam, clonazepam, lorazepam, lormetazepam, zopiclone, flurazepam, nordiazepam, triazolam, alprazolam, zolpidem, ketamine, and nitrazepam D5 were obtained from *Cerilliant* (Austin, TX, USA). Scopolamine was from *ChromaDex* (Irvine, CA, USA). Formic acid was from *Riedel-de-Haën* (Seelze, Germany). Bromazepam, acetonitrile (HPLC grade), dichloromethane, and methanol were provided by *Sigma-Aldrich* (St. Louis, MO). Ultrapure water was obtained using a *Milli-Q®* UF-Plus apparatus (*Millipore*, Bedford, MA, USA). Every day stock solutions of drugs and nitrazepam D5 were diluted with methanol to obtain working solutions at 100 ng/mL.

Sample collection and preparation

Hair was cut close to the scalp from the vertex posterior area of each subject's head. Extremities corresponding to the cut were marked in all collected strands. Hair from woman #1 was thin, bleached (blond), and 16 cm long, while hair from woman #2 was thick, non-treated (black), and 27 cm long. Hair from the suspect and his wife were non-treated, black, and 5 and 18 cm long, respectively. Samples were stored at room temperature in plastic tubes.

One strand was used for hypnotic drug screening, one for cannabis testing (only for woman #1), and the third one was kept dry for potential counter analysis. About 100 mg of each hair segment was washed twice with dichloromethane (3 mL, vortex mixed for 3 min). After complete removal of the solvent wash, the hair was dried at room temperature by a gentle nitrogen flow and then cut with scissors into 1–2-mm segments.

A 2-mL aliquot of methanol was added to the crumbled hair. Deuterium-labeled nitrazepam was added as the internal standard at a final concentration of 50 pg/mg. The samples were incubated at 55°C for 15 h. Lastly, the organic phase was collected and evaporated to dryness at 55°C. The residue was reconstituted in 50 µL of liquid chromatography (LC) mobile phase (5 mM formic acid buffer to acetonitrile, 90:10; v/v).

The detection of Δ^9 -tetrahydrocannabinol and its metabolites in urine, blood, and hair is not described in detail here. These determinations are indeed part of routine analyses performed in all the forensic toxicology laboratories.

LC-MS/MS procedure

LC was performed using an Agilent 1100 series instrument. A 15-µL aliquot of the extract was injected into the column (Phenomenex Synergi Fusion-RP 150×2.00 mm i.d.×4 µm), protected by a C18 guard column. The 15-min LC run was carried out using a gradient (from 10% acetonitrile to 100% in 15 min) at a flow rate of 250 µL/min. Mass detection was performed by an AB Sciex API 4000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in the positive ion mode. Best results were obtained by utilizing a source block temperature of 350°C. Data were recorded in the selected reaction-monitoring (SRM) mode. In order to establish the most appropriate SRM conditions, all target substances were individually infused into the ESI capillary and the MS entrance cone voltage was adjusted so as to maximize the intensity of the protonated molecular species $[M+H]^+$. Optimized chromatographic and mass-spectrometric conditions for targeted molecules and IS are presented in Table 1.

Electron microscopy

For scanning electron microscopy (SEM) observations, intermediate section (segment from 2 to 3 cm for the reference standard and segment from 5 to 6 cm for woman #1) of hair samples were deposited on an aluminum stub covered with a bi-adhesive conductive carbon tape, and sputtered with Au, to form a surface-conductive layer ca. 30 nm thick (BAL-TEC Sputter-Coater, ModelSCD 050). Images

were obtained with a Leica Stereoscan 420 microscope 20 kV by collecting secondary electrons (E-T detector) emerging from the samples under the following operational conditions: acceleration potential, 20 kV; beam current, 60 µA; and I probe, 80 pA.

Results and discussion

Method validation

The method validation was carried out according to 2006 SOFT/AAFS guidelines [13]. Selectivity, linearity range, and identification and quantitation limits [limit of detection (LOD) and limit of quantification (LOQ)] were determined. The matrix effect was also evaluated [14]. Validation data are reported in Table 2. The absence of any significant carry-over effect was positively verified by running a blank sample after processing the highest calibration level. All analytes were eluted within a 15-min ramp. The total analysis turnaround time including re-equilibration was 22.0 min.

Selectivity and identification criteria

A pool of five different negative hair samples was prepared and analyzed as described above. For each sample and all the analytes, the signal-to-noise ratio was measured for the main mass transitions at the expected retention time windows. No interferences were observed from the pooled negative blank hair used for validation. The repeatability of relative peak intensities for each analyte transitions was determined from five spiked hair samples at the concentration of 25 pg/mg. Three SRM transitions selected for each analyte provided at least four identification points, while the substantial stability of their relative abundances proved compliant for the unambiguous identification of all analytes included in the assay, in agreement with CE/2002/657 decision and 2006 SOFT/AAFS guidelines criteria.

Linearity, limit of detection, and limit of quantitation

Standard calibration curves were prepared from blank hair fortified with standard solutions at final concentrations of 2, 5, 10, 25, 50, and 100 pg/mg plus a “zero” sample. Calibration lines were checked for linearity using the least squares regression method. Adequate linearity was observed for all compounds. The resulting correlation coefficients (R^2) are reported in Table 2. Quantitative data resulting from area counts were corrected using the IS signal areas. The LOD was calculated as the analyte concentration yielding a signal (peak area) equal to the

Table 1 Precision of retention time (coefficient of variation, in percent) and MS transitions of the screened compounds

Compound	RT (min)	RT precision ($n=10$) CV% 25 pg/mg	SRM transitions (m/z)
Scopolamine	8.09	0.16	304.3→156.3 304.3→121.3 304.3→138.3
Ketamine	9.00	0.20	238.0→220.3 238.0→179.1 238.0→138.3
Zopiclone	9.68	0.12	389.3→245.2 389.3→345.3 391.3→247.3
Zolpidem	10.49	0.11	308.2→236.4 308.2→263.3 308.2→235.3
Flurazepam	11.35	0.25	388.3→315.3 388.3→317.3 390.3→317.3
Bromazepam	11.98	0.19	316.1→209.1 316.1→182.3 318.1→182.4
Lorazepam	13.37	0.17	321.1→275.2 321.1→229.2 323.1→277.1
Nordiazepam	13.41	0.09	271.1→140.1 271.1→165.0 271.1→208.3
Clonazepam	13.67	0.14	316.2→241.3 316.2→214.2 316.2→154.3
Alprazolam	14.04	0.13	309.3→281.3 309.3→274.2 309.3→205.1
Flunitrazepam	14.12	0.12	314.2→268.2 314.2→212.2 314.2→286.7
Triazolam	14.19	0.33	343.2→308.2 343.2→315.0 345.1→317.2
Lormetazepam	14.26	0.22	335.2→289.2 335.2→350.2 337.3→291.2
Diazepam	14.64	0.17	285.3→222.4 285.3→193.2 285.3→154.3
Nitrazepam-D5	13.20	0.10	287.3→241.3 287.3→212.2 287.3→185.5

RT retention time, CV% percent variation coefficient, SRM selected reaction-monitoring mode

average background (S_{blank}) plus three times its standard deviation S_{blank} ($\text{LOD}=S_{\text{blank}}+3s_{\text{blank}}$), while the LOQ is

given as $\text{LOQ}=S_{\text{blank}}+10s_{\text{blank}}$. For each analyte, LOQ generally corresponds to the lowest concentration that

Table 2 Validation data for the screened molecules

Compound	Calibration levels (pg/mg)	Correlation coefficient (R^2)	Matrix effect ($\pm\%$)	LOD (pg/mg)	LOQ (pg/mg)	Precision (CV%)	Accuracy bias (%)
Scopolamine	5–100	0.991	−7.1	0.7	2.3	9.0	+4.4
Ketamine	2–100	0.996	+13.2	0.2	0.7	11.6	+3.0
Zopiclone	5–100	0.997	+5.1	0.9	3.0	6.8	+2.7
Zolpidem	2–100	0.994	−4.7	0.2	0.7	11.0	+3.3
Flurazepam	2–100	0.995	+2.6	0.5	1.7	12.9	−1.1
Bromazepam	10–100	0.992	+8.2	2.0	6.7	14.0	+0.5
Lorazepam	25–100	0.992	−4.9	3.2	10.6	12.7	+2.8
Nordiazepam	5–100	0.997	+8.9	1.2	4.0	2.3	+1.8
Clonazepam	2–100	0.995	+1.2	0.6	2.0	5.7	+1.5
Alprazolam	10–100	0.990	−6.3	1.8	5.9	2.8	+2.3
Flunitrazepam	25–100	0.993	−8.2	4.0	13.2	8.2	+2.5
Triazolam	5–100	0.996	−17.3	0.7	2.3	18.1	+1.3
Lormetazepam	10–100	0.989	+4.6	2.4	7.9	12.4	+2.7
Diazepam	5–100	0.994	−16.9	0.5	1.7	8.5	+3.1

LOD limit of detection, LOQ limit of quantification, CV% percent variation coefficient

provides a useful signal along the calibration curve. Table 2 reports LOD and LOQ values, calculated from the analysis of multiple blank samples and confirmed (LODs) experimentally. LOD values ranged between 0.2 and 4.0 pg/mg while LOQ values were calculated between 0.7 and 13.2 pg/mg. Calibration levels below LOQs were excluded from the curve.

Matrix effect, precision, and accuracy

The matrix effect was determined for each molecule by comparing the representative chromatographic peak area obtained from hair samples, spiked at the final concentration of 25.0 pg/mg, with the peak area of a methanolic standard at the same concentration. Within-batch precision

(expressed as percent variation coefficient, CV%) and accuracy (expressed as bias percent), were assessed by extracting and analyzing a series of five hair samples spiked at 25.0 pg/mg. The data are reported in Table 2.

Analyses of real samples

Woman #1

The blood and urine samples obtained from the 26-year-old Caucasian alleged victim showed past tetrahydrocannabinol (THC) consumption. Blood concentration of 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) was 7.4 ng/mL, while negative results were observed for the parent drug and the 11-hydroxy- Δ^9 -tetrahydrocannabinol

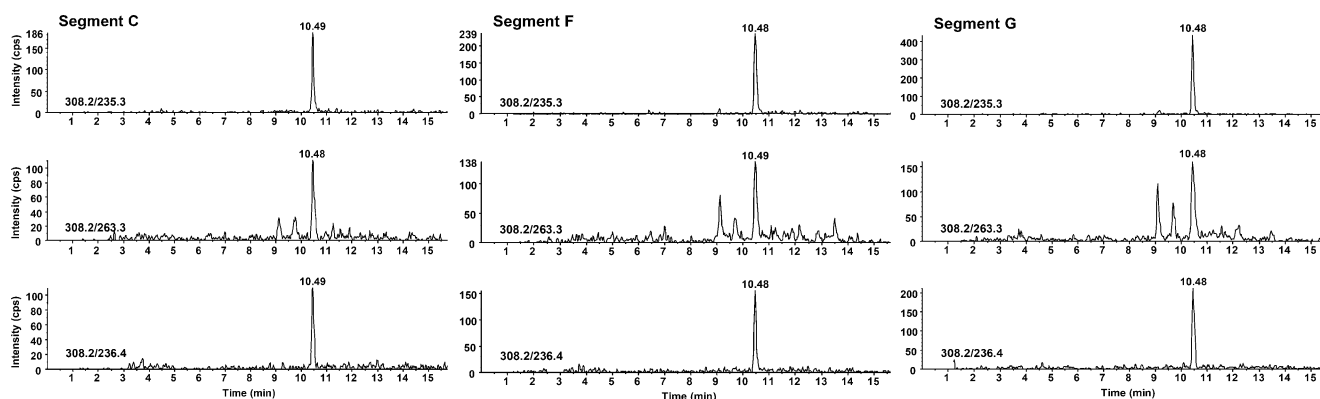


Fig. 1 SRM chromatograms of segments C, F, and G positive for zolpidem (woman #1)

Table 3 Results of segmental hair analysis in woman #1

Segment	Diagnostic window (estimated average hair growth, 1 cm/month)	Length (cm)	Findings (pg/mg)
A	October 2009–November 2009	1	Δ^9 -THC (24)
B	September 2009–October 2009	1	Δ^9 -THC (24)
C	August 2009–September 2009	1	Zolpidem (2.8) Δ^9 -THC (50)
D	July 2009–August 2009	1	Δ^9 -THC (56)
E	June 2009–July 2009	1	Δ^9 -THC (47)
F	May 2009–June 2009	1	Zolpidem (1.6)
G	March 2009–May 2009	2	Zolpidem (0.9)
H	January 2009–March 2009	2	Δ^9 -THC (166)
I	November 2008–January 2009	2	
L	September 2008–November 2008	2	
M	July 2008–September 2008	2	

THC tetrahydrocannabinol

metabolite. Urinary THC-COOH was determined at a concentration of 108 ng/mL. These results could only prove past exposure to *Cannabis sativa*, but no conclusion could be drawn about a possible impairment related to its consumption at the moment of the alleged assault. Detectable levels of THC on the entire hair length proved that the woman was a regular consumer of *C. sativa*.

From the screening for benzodiazepines and analogues, hair results evidenced the presence at trace level of the non-benzodiazepinic hypnotic zolpidem in the different segments (segments C, F, and G). Among these three, it is worth noting that two segments are consecutive. Evidence of the analytical findings for segments C, F, and G is shown in Fig. 1, while the summary of hair analysis results is reported in Table 3. The total hair length was 16 cm; zolpidem was found in segment 2–3 cm (concentration

2.8 pg/mg); segment 5–6 cm (1.6 pg/mg); and segment 6–8 cm (0.9 pg/mg). As a preliminary evaluation, the low picogram/milligram concentration range found in each segment is coherent with episodic exposure to the drug, possibly single exposure in two–three different periods. Assuming that the positive results found in the F and G consecutive segments (from 5 to 8 cm) may arise from a single assumption, it was deduced that zolpidem was ingested in at least two occasions, whereas it was excluded that the woman was a regular consumer of the drug.

A further aspect taken into account during the present investigation was the possible hair structure deterioration occurring by thermal or cosmetic treatments. In such cases, hair swelling and water absorption capacity increase, producing a displacement by radial migration of the drug along the hair cross-section, after its incorporation into the

Fig. 2 SEM of (A, A') non-treated hair sample; (B, B') hair sample from woman #1. Original magnifications, images A, B ($\times 1,500$); images A', B' (zoom of the previous ones; $\times 3,500$)

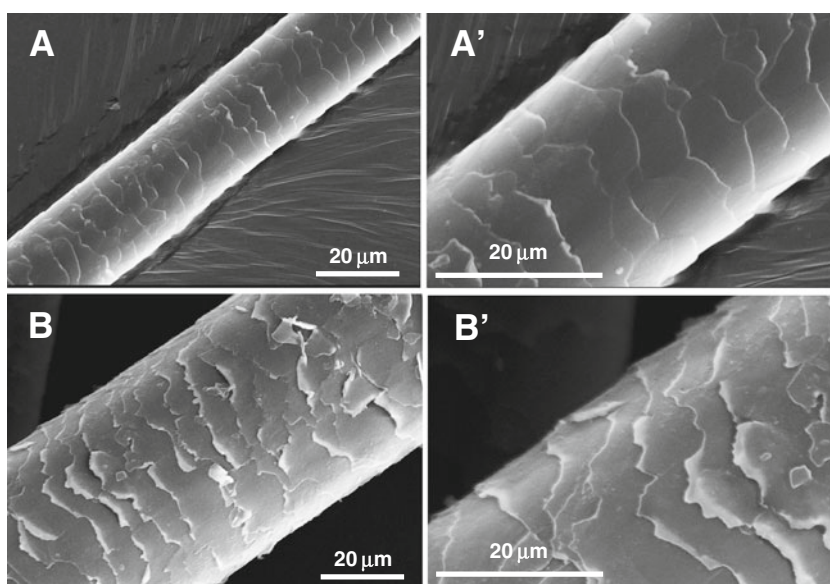


Table 4 Results of segmental hair analysis in woman #3

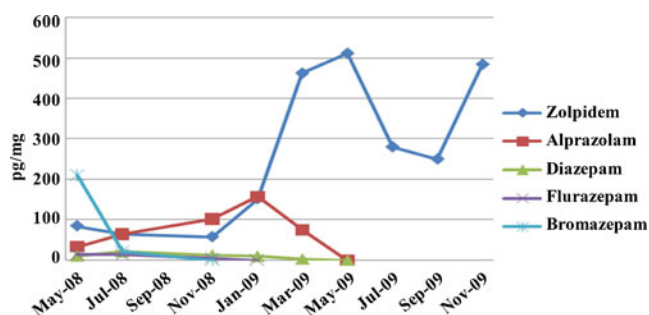
Segment	Diagnostic window (estimated average hair growth, 1 cm/month)	Length (cm)	Findings (pg/mg)
A	September 2009–November 2009	2	Zolpidem (484)
B	July 2009–September 2009	2	Zolpidem (250)
C	May 2009–July 2009	2	Zolpidem (280)
D	March 2009–May 2009	2	Zolpidem (511)
E	January 2009–March 2009	2	Zolpidem (462) Alprazolam (74.7) Diazepam (2.6)
F	November 2008–January 2009	2	Zolpidem (152) Alprazolam (157) Diazepam (9.9) Flurazepam (traces)
G	September 2008–November 2008	2	Zolpidem (58.3) Alprazolam (102) Diazepam (13.3) Flurazepam (5.0)
H	July 2008–September 2008	2	Zolpidem (65.2) Alprazolam (64.4) Diazepam (20.8) Flurazepam (14.5) Bromazepam(22.3)
I	May 2008–July 2008	2	Zolpidem (85.5) Alprazolam (33.0) Diazepam (10.6) Flurazepam (14.1) Bromazepam(211)

matrix [15]. In order to verify the degree of hair degradation which may lead to a partial loss of the drug, microscopic analysis was performed. A black, non-treated hair was selected as a reference standard and compared to the hair of woman #1, as shown in Fig. 2. The evident alteration of the keratinic structure of hair collected from woman #1 supports the hypothesis of partial drug loss after abundant water absorption into the hair, especially in concurrence with special conditions such as intense sweating. On the other hand, axial migration of the drug

along the hair length appears not to be favored by hair degradation. Thus, the presence of the drug in two consecutive segments may arise from the hair growth cycle. In particular, it is known that broadening of xenobiotics along the hair length may be produced during the resting period of hair growth [16].

Woman #2

Hair analysis from the 41-year-old African alleged victim produced negative results for all the screened molecules, except for diazepam, present at low concentration level (from the traces of 9.6 pg/mg). Positive detection of diazepam in various biological samples is often of problematic interpretation because alternative explanations exist other than drug use. First, it has been shown in the past that the serum of unmedicated humans may contain diazepam together with its main active metabolites at concentrations of 1–32 ng/L, possibly as the result of benzodiazepines naturally being present in certain foods [17–19]. Secondly, the presence of diazepam without metabolites does not provide unequivocal evidence of diazepam exposure. Once administered, diazepam is exten-

**Fig. 3** Results of segmental hair analysis in woman #3: administration profile of different hypnotic drugs

sively metabolized in blood to nordiazepam. Thereafter, both compounds are converted to their 3-hydroxy-derivatives (temazepam and oxazepam), which represent the main urinary metabolites [20–22]. Whenever diazepam is administered, other metabolites are present in the biological fluids, but at least the main blood metabolite nordiazepam is likely to be detected in hair. It was demonstrated that after a controlled administration of a single dose of Valium® (diazepam) to three volunteers, hair samples were positive for diazepam (2.3–6.0 pg/mg) and nordiazepam (traces—5.4 pg/mg) [23]. In our laboratory, one hair sample from a chronic user and one from a volunteer after a single administration were recently analyzed. The results indicated 4,197 pg/mg of diazepam and 3,054 pg/mg of nordiazepam, respectively, for the chronic user and the presence of both diazepam and nordiazepam at comparable trace level for the volunteer (unpublished data). In the present case, we observed that (a) the hair concentration of diazepam was extremely low (<10 pg/mg) and (b) constant in all segments, moreover (c) diazepam metabolites were totally absent along the entire length of the hair. It was concluded that the analytical signals attributed to the presence of diazepam could alternatively be due to co-eluting interference, so we were not confident from hair analysis that diazepam had actually been taken. Another element of uncertainty is the long period of time (17 months) elapsed between the alleged crime and hair collection. Although xenobiotics are known to be trapped into the hair structure for extended periods, it is not unlikely that the most hydrophilic ones (i.e., some metabolites) could be progressively washed out from the keratin matrix.

Person under investigation

The analysis of hair samples collected from the man proved negative for all the screened molecules.

Woman #3

The analysis of hair collected from the suspect's wife showed the presence of various drugs at high concentration levels. The complete results are shown in Table 4. Figure 3 reports a detailed graphical representation of the various hypnotic drug abundances during the approximate period explorable by hair analysis. These results confirmed that the woman made a therapeutic use of zolpidem at increasing dosage, while, at the same time, the administration of other hypnotic drugs was gradually reduced. It was concluded that (a) all the hypnotic drugs consumed by woman #3 were for therapeutic purpose and (b) the availability of zolpidem in the suspect's house was abundant and easily accessible.

Conclusions

Many hypnotic drugs are effective agents for drug-facilitated sexual assaults, due to their wide and easy availability, low efficacious dose, and various pharmaceutical forms, facilitating clandestine administration. The basic pharmacology of zolpidem—showing rapid onset of action, effectiveness to induce and maintain sleep/unconsciousness, and amnesic properties—also makes this drug an effective DFSA agent [24]. In fact, zolpidem is becoming one of the most frequently encountered substances in drug-facilitated crimes, so that the interpretation of its presence in biological samples, especially in hair analysis, has become a current toxicological challenge.

The detection of zolpidem and other so-called rape drugs at very low concentration in hair samples may represent single or occasional exposure to the drug, while higher levels usually indicate regular and therapeutic administration, although there is no inter-individual correlation between the frequency and dose of drug intake and hair concentration [16]. Furthermore, the approximate period of drug exposure can be estimated by the distance of the positive hair segment from the scalp provided that (a) uniform and rather constant hair growth rate can be assumed and (b) the incorporation of the drug occurs only within the hair root. Lastly, the presence of confounding interferences in the hair matrix, or changes in the hair structure due to heavy cosmetic treatments, might mislead the final result of hair analysis.

The case described in the present study, in which various complex issues were simultaneously encountered, encouraged our toxicological laboratory to follow strict rules in executing hair analysis experiments and interpreting the meaning and significance of analytical findings as follows: (a) always perform a detailed segmental analysis in order to compare the drug concentration results, enabling occasional exposure from regular administration to be distinguished; (b) include direct drug metabolites in the panel of the screened substances in order to confirm or exclude alternative explanations for drug detection; and (c) whenever possible, carry out a microscopic investigation of the physical state of the hair, particularly when the drug was detected at very low concentration (i.e., the typical DFSA conditions) in order to take into account the chance of drug loss or contamination from an external source.

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