Int J Legal Med (2012) 126:637–643
DOI 10.1007/s00414-011-0654-6

CASE REPORT

A drug rape case involving triazolam detected in hair and urine

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Received: 2 September 2011 / Accepted: 24 November 2011 / Published online: 9 December 2011

Abstract In recent years, there has been heightened awareness regarding the use of drugs to modify a person's behavior to facilitate crime. A drug rape case involving the potent, short-acting sedative triazolam will be presented. On three occasions, the victim consumed green tea and chocolate before being massaged and ultimately sexually abused. Screening for alcohol, commonly used drugs and illicit substances in blood and urine sampled during the forensic examination 20 h after the last incident, was negative. Consequently, hair samples for chemical analysis were taken from the assaulted individual 34 days after the last incidents. The hair was cut into three 2-cm segments (0–6 cm) that were washed, dissolved in extraction solvent and screened and verified by ultra performance liquid chromatography coupled with time of flight mass spectrometry (UPLC-TOF-MS) and with tandem mass spectrometry (UPLC-MS/MS), respectively. In the 2-cm hair segment corresponding to the period of the alleged assaults, the presence of the sedative triazolam was revealed at a concentration of 1.0 pg/mg hair. The preserved urine sample, taken 20 h after the last incident, was reanalyzed by UPLC-MS/MS for metabolites of triazolam, and 39 μg/l α-hydroxytriazolam was detected in the hydrolyzed urine. This case illustrates that hair is a valuable forensic specimen in situations where natural processes have eliminated the drug from typical biological specimens due to delays in the crime being reported. Furthermore, it was possible to verify the hair finding with a urine sample by detection of a metabolite of triazolam.

Keywords Hair · Urine · Drug-facilitated crime · Triazolam and metabolite α-hydroxytriazolam

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Introduction

Hair has become a good alternative or supplemental matrix to blood and urine. It is a more stable matrix and has a substantially longer detection window, from weeks to years. For this reason, hair has proven to be an excellent matrix in, e.g., drug-facilitated crimes (DFC) [1–3]. Segmental hair analysis, in particular, provides useful information on the state and history of the drug use [4], enabling the differentiation between single exposure and chronic use. The challenge, however, is that a single-dose exposure is difficult to detect because the target concentrations in hair are very low [5]. Effective extraction and sensitive detection of drug rape substances, such as the benzodiazepines, from hair samples is crucial due to the low levels of drugs that are incorporated into the hair. Highly sensitive and specific analytical techniques, such as liquid chromatography-mass spectrometry (LC-MS)/MS, are therefore recommended for trace-level identification and quantification of drug rape substances [4, 6].

This paper illustrates the potential of hair analysis for DFC involving triazolam and quantifies the level in hair, which to our knowledge have not been presented before.

Case

According to the victim, a 30-year-old woman, the suspect had encouraged her to try a purportedly deep-cleansing

procedure consisting of a whole-body massage preceded by the consumption of chocolate and green tea. This "ritual" took place on three separate occasions: at 17 and 7 days and finally approximately 20 h prior to the victim's forensic examination. The suspect had repeatedly cautioned the victim that she might feel drowsy, possibly even fall asleep, after the massage. Only on the first of the three occasions did the victim participate in the preparation of the chocolate and green tea. The first encounter was uneventful. During the second and third encounters, she recalled feeling lethargic and dizzy but attributed this solely to the effects of the massage. Even though she had explicitly declined the suspect's offer to massage her in intimate areas of her body, she felt indifferent when he did so. Similarly, she did not resist when he sexually assaulted her.

According to the victim, she had consumed a small glass of red wine half an hour before the final encounter. There had been no recent use of medication or illicit substances. Twelve hours after the final encounter, the victim consulted her general practitioner who performed a multi-drug screen urine test (unknown manufacturer) that was positive for a benzodiazepine class drug. Regrettably, this urine sample was discarded and thus unavailable for forensic analysis. Upon forensic examination, a bluish/yellowish bruise was found on the left upper arm and a superficial abrasion on the left side of the back. The gynecological exam was unremarkable. Fluorescence was demonstrated in two areas by ultraviolet light: on the medial aspect of the right upper leg and on the lateral aspect of the left labium majus.

Toxicological analysis was performed on blood and urine samples taken 20 h after the last encounter. Blood and urine alcohol content was negative as was screening of blood and urine for common drugs (sedatives, painkillers and psychoactives), illicit substances including γ-hydroxybutyrate (GHB) and analogs. Consequently, hair was sampled from the back of the victim´s head 34 days after the last encounter. The victim had dyed her hair a few days prior to sampling.

Materials and methods

Triazolam from Pfizer (Ballerup, DK) and α-hydroxytriazolam from Upjohn (Gentofte, DK) were used as reference materials, and stock solutions at 1,000 mg/l were prepared in methanol. Deuterated D_4-triazolam was used as internal standard (IS) for urine analysis, while D_5-diazepam was used for hair (deut. triazolam was not available at the time of the hair analyses), both from Cerilliant (Round Rock, TX, USA). Acetonitrile and methanol of LC-MS grade were obtained from Fisher Scientific (Leicestershire, UK) and formic acid 98–100% grade, butyl acetate for analysis and sodium hydroxide were supplied from Merck (Darmstadt, DE). Purified water was obtained from Millipore Synergy UV water purification system (Millipore A/S, Copenhagen, DK). The extraction medium (EM) was a 25:25:50 (v/v/v) mixture of methanol/acetonitrile/2 mM ammonium formate containing 8% acetonitrile (pH 5.3). β-Glucuronidase/arylsulfatase (*Helix pomatia*) was obtained from Roche Diagnostics (Copenhagen, DK). Drug-free human hair and urine were obtained from six laboratory volunteers and their children.

Analytical method

Hair

The screening method was performed according to the procedure described by Nielsen et al. [7], i.e., 10 mg cut hair (1–2 mm) with some modifications. We reduced the volume of EM to 250 μl and the injection volume was doubled to 10 μl in order to increase sensitivity. Changing the analytical method from time of flight (TOF)-MS to MS/MS resulted in a several-fold increase in sensitivity. For confirmation analysis, 20 mg of washed, segmented hair was used (instead of the aforementioned 10 mg), and the EM volume was further reduced to 150 μl. The segmented hair was cut into small pieces of 1–2 mm and then washed, once with 2 ml 2-propanol in an ultrasonic bath for 5 min at room temperature, and then 3×5 min with 1 ml aqueous solution. Calibration standards in hair (0.3, 0.6, 0.9, 1.5, 15, 150, 750 pg/mg) were prepared by adding dilutions of the stock solution in EM to blank hair. Two quality control (QC) samples in hair at 100 and 700 pg/mg were prepared as described by Nielsen et al. [7] containing triazolam and other benzodiazepines. These were analyzed together with the samples. Twenty microliters of 0.20 mg/l IS (D_5-diazepam) in EM was added to the samples together with 150 μl EM. The samples were centrifuged at 3,600×*g* for 10 min at 20°C to ensure that all hair pieces were in the EM before being incubated at 37°C for 18 h in an oven [7]. The hair samples were then centrifuged for 10 min and the solution was transferred to brown mini-UniPrep vials containing PTFE filter (Whatman Inc., Piscataway, NJ). Finally, 10 μl of each filtered extract was injected onto the UPLC-MS/MS. To check for external contamination, the aqueous wash fractions were also analyzed on the UPLC-MS/MS as described below.

Urine and wash fractions

Urine samples were centrifuged for 10 min and the upper layer was amended for analysis. Urine (500 μl) was added 0.25 ml 1 M acetate buffer for pH 5.5 and then 25 μl β-glucuronidase/arylsulfatase solution (diluted 1:3 with water). This mixture was incubated for enzymatic hydrolysis 18 h at 40°C. One hundred microliters of centrifuged

urine or wash fractions were spiked with 20 µl of 50 µg/l IS (D_4-triazolam) in water following the addition of 150 µl water, 50 µl 2N NaOH and 250 µl butylacetate. After having been mixed thoroughly, the organic phase was transferred to another glass and evaporated under a stream of nitrogen at 40°C. The remains were reconstituted in 200 µl solvent (1:4:5 acetonitrile/methanol/acidic water), and the solution was subsequently moved to an amber vial. Calibration standards in urine (2, 10, 50, 200, 500 µg/l) were prepared using dilutions of the stock solution in water. Five microlitres of each extract was injected onto the UPLC/MS/MS system.

Quantification and confirmation in hair and urine were performed by chromatographic separations on a 100× 2.1 mm ACQUITY BEH 1.7 µm C_{18} column (Waters Corp., Milford, MA, USA) using an ACQUITY Ultra Performance Liquid Chromatography system (Waters Corp.). The mobile phase was composed of solvents A: 0.05% formic acid and B: 0.05% formic acid in acetonitrile. The column was maintained at 50°C and eluted with a linear gradient of 10–20% B (0–4.0 min), 20–36% B (4.0–11.0 min), 36–72% B (11.0–11.1 min), 72% B (11.1–12.0 min), before returning to 10% solvent B. The total runtime was 16.5 min to ensure re-equilibration between injections at a flow rate of 0.60 ml/min. The autosampler was maintained at 12°C.

Mass spectrometry was performed on a tandem quadrupole ACQUITY TQD (Waters Corp.). Ionization was achieved by using electrospray in the positive mode (ESI+). Mass spectrometer conditions were optimised by infusion of a standard solution through a T-piece into the Z-spray ion source while mixing with a continuous flow of mobile phase (0.6 ml/min, 50% solvent B). The optimised parameters were: capillary voltage, 1.00 kV; extractor voltage, 3.00 V; desolvation gas flow, 1,100 l/h; collision gas flow, 0.18 ml/min argon at 0.004 mbar; desolvation temperature, 450°C; source temperature, 120°C. Data acquisition was done in multiple reactions monitoring (MRM) mode. The optimised MRM transitions (two per target compound), cone voltages and collision energies are given in Table 1. The identification parameters for the target analytes were absolute retention time (RT), relative retention time against IS (RRT), MRM traces, and ion ratio (MRM2/MRM1). The criterion for ion ratio was set to ±10% from the mean of the calibration standards, and the criteria of RT and RRT were set to ±0.03 and ±0.01 min from the mean of the calibration standards, respectively.

Results

Analysis and validation parameters

Selectivity of the system was tested by injection of standard solutions at 1 mg/l of approximately 50 common pharmaceuticals and drugs of abuse. No interference was detected. Furthermore, six hair and urine samples from volunteers were found negative. The system is applied for the determination of common benzodiazepines and other basic pharmaceuticals in hair, so the method´s potential is wide. The recovery of triazolam and its metabolite α-hydroxytriazolam was 80% and 65% in hair, and 105% and 60% in urine, respectively. There was a wide linear range, from 0.3 to 750 pg/mg hair, for triazolam with deviations within ±15% and a correlation coefficient above 0.99. Limit of detection (LOD) was estimated at 0.1 pg/mg and the limit of quantification (LOQ) at 0.3 pg/mg for triazolam**.** The imprecision (CV) of the QC hair samples was less than 15% and the bias was below 15%. Ion ratio and identification criteria of triazolam were 1.5±10%. The accuracy assessed by comparing the QC values of this method with the method described by Nielsen et al. [7], as no commercial hair reference so far exists for this drug, was determined to be better than 85%. The metabolite had a linear range from 1.5 to 750 pg/mg hair, while the LOD and LOQ were five times higher than its main drug. In urine, the linear range was from 2 to 500 µg/l with deviations within 20% for both compounds. The LOD was estimated to be 1/10 of LOQ at 2 µg/l as this had S/N levels of about 300 for both compounds. Ion ratio of the metabolite was 3.6, as shown in Table 1.

The quantitative method met the necessary sensitivity described in the literature for these types of drugs (benzodiazepines) in date rape cases/single dose applications [7, 8]. For instance, Sun et al. [8] described a LC-MS/MS method for a single dose of triazolam in guinea pig hair with LOD of about 1 and 5 pg/mg of triazolam and metabolite, respectively. The method presented here is more sensitive than that presented in the study of Sun et al. [8].

Table 1 Parameters of the quantitative method

Compound	RT (min)	Cone (V)	Coll1 (eV)	Coll2 (eV)	MRM1 *m/z*	MRM2 *m/z*	Ion ratio
Triazolam	9.59	50	27	40	343→308	343→239	1.5
α-Hydroxytriazolam	8.47	45	27	27	359→141	359→176	3.6
D_5-Diazepam	10.67	40	35	–	290→198	–	–
D_4-Triazolam	9.58	56	29	–	347→312	–	–

Case study

Hair

An initial screening of the innermost 2 cm of an unwashed scalp black hair sample collected from the victim demonstrated traces of triazolam. A second scalp hair sample was cut into three segments of 2 cm each, corresponding in time to approximately the previous 6 months. The segmental analyses followed general guidelines [5]. For instance, all segments were washed accordingly. In the first (inner) segment, corresponding to the most recent 2 months, triazolam was detected at a concentration of 1.0 pg/mg hair, while the other two segments (2–6 cm) were negative, as shown in Table 2. Ion chromatograms of a hair blank (a), a low hair standard (0.3 pg/mg; b) and hair from the case (c) are shown in Fig. 1. The small trace of triazolam in the quantitative trace on the hair blank was several-fold below LOD, and the ion ratio criteria were not fulfilled either. No metabolite of triazolam was detected in the hair samples. No carry-over was determined within the system. Additionally, to exclude the possibility of external contamination of the hair, the aqueous wash fractions per hair sample were analyzed and found negative for triazolam as well as for the metabolite. Due to the narrow time frame, it was not possible to separate the two incidents within the hair analysis.

Urine

In the urine sampled 20 h after the last incident, the triazolam metabolite α-hydroxytriazolam was detected at 39 μg/l, as shown in Fig. 2, while the blank urine was negative. α-Hydroxytriazolam is an active metabolite that is glucuronised in urine and released following glucuronidase treatment before the extraction and analysis. This detection concurs with the findings in the hair. The metabolite α-hydroxytriazolam is now included in our screening method for future investigations.

In court, it was revealed that the suspect had been prescribed triazolam by his general practitioner. The suspect confessed to having given the victim triazolam without telling her but denied charges of rape. He was found guilty of committing drug-facilitated rape and was sentenced to prison.

Table 2 Results (mean of double determination) of segmented hair and urine sample from the victim

Compound	Segment 1 0–2 cm (pg/mg)	Segment 2 2–4 cm (pg/mg)	Segment 3 4–6 cm (pg/mg)	Urine (μg/l)
Triazolam	1.0	N	N	N
α-Hydroxytriazolam	N	N	N	39
IS (recoveries, *n*=2)	105%	104%	85%	94%

N negative

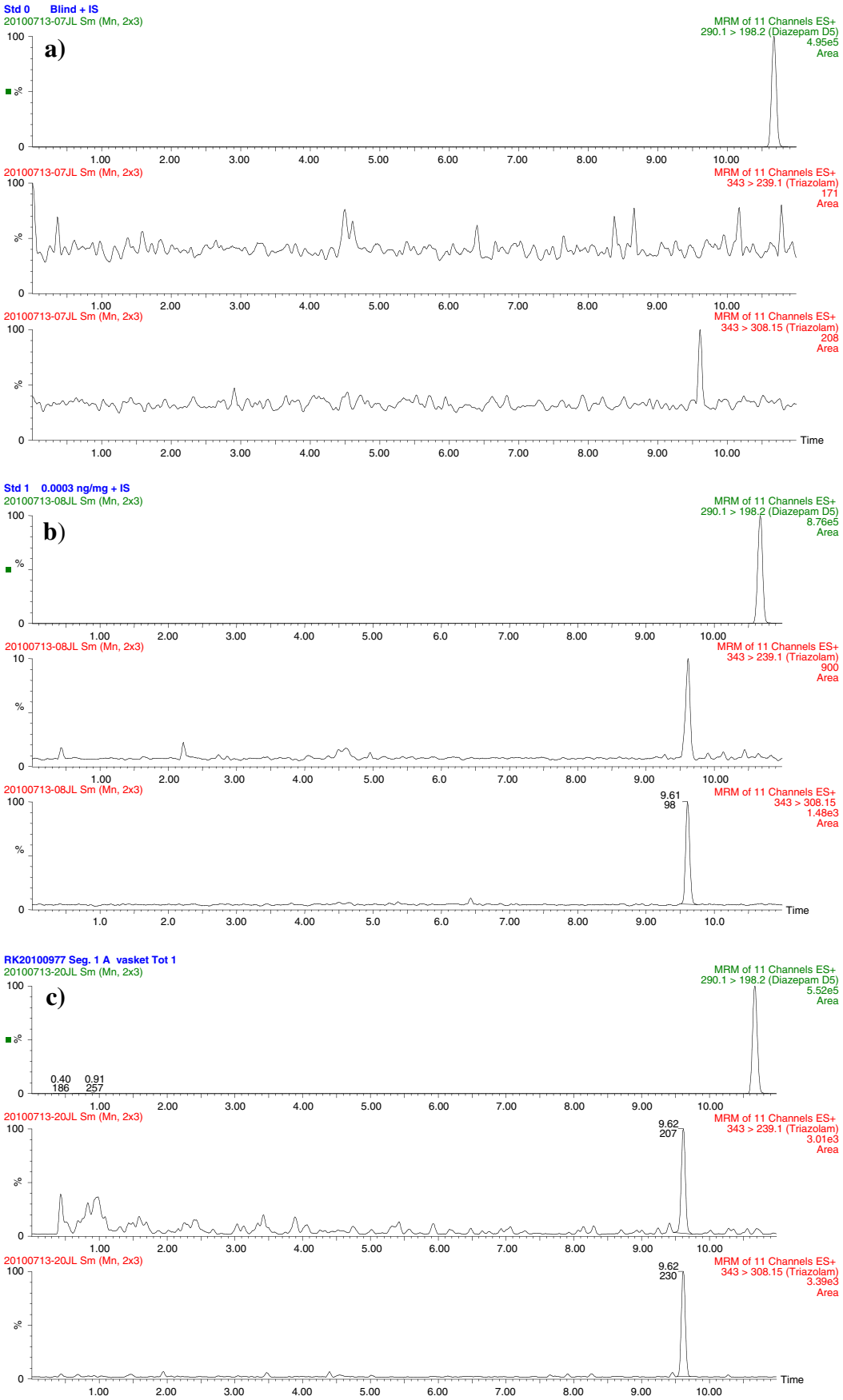
Discussion

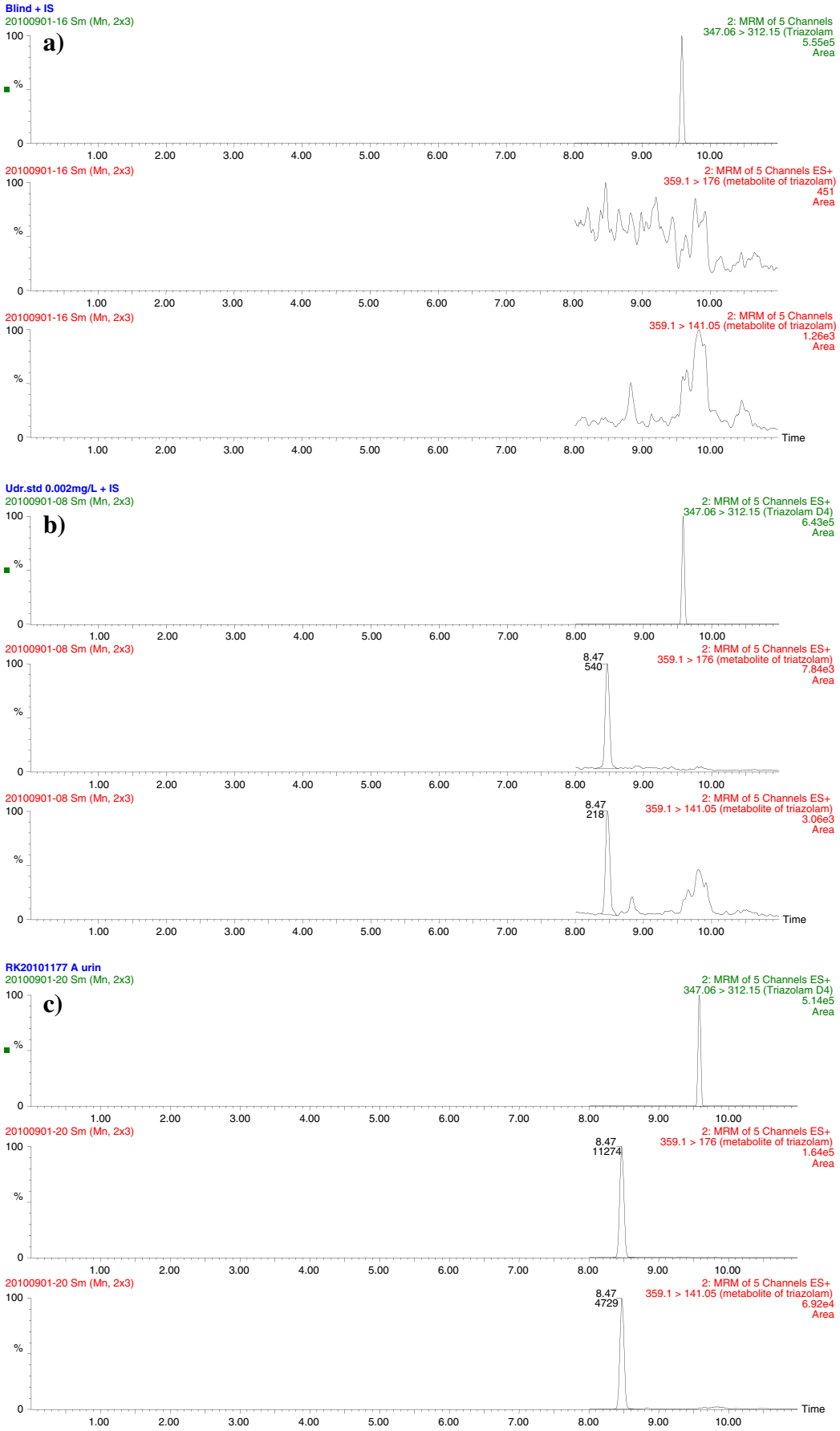
Many published drug rape cases involve benzodiazepines and benzodiazepine-like "Z" compounds such as clonazepam, bromazepam, midazolam and zolpidem [9–11]. In contrast, triazolam findings in drug rape cases are rare [9, 11] and the concentration has never been reported before. This might be due to the drug's very short half-life (a few hours — the shortest half-life of all the orally administered benzodiazepines), sampling delay and lack of sensitive methods [7, 11]. In addition, hair sampling is rarely performed. Furthermore, the drug use is limited as other benzodiazepines are preferred and in some countries therapeutic prescription of triazolam has been withdrawn due to its adverse effects making the availability of the drug to the public low.

Toyo'oka et al. [12] presented the case of a drug addict in whom both triazolam and metabolites were detected in the hair, but the drug use was high in that study.

The level detected in this particular hair case indicated low intake of triazolam when compared to other similar publications [8, 9] although these cases involved administration of triazolam to pigs or prescribed sedative (triazolam) with unknown use/compliance. It is possible that the dark hair of this case improved the detection possibilities since the high melanin content improves absorption of basic drugs such as triazolam. On the other hand, it should be noted that the victim dyed her hair after the described incidents and thereby probably reduced the amount of drug in her hair. The metabolite was not detected in the hair, a finding which could be attributed to the higher LOQ of this more polar compound and lower degree of incorporation of the metabolite into hair. Sun et al. [8] found higher concentrations of the metabolite in guinea pig hair than the main drug triazolam. However, their data interpretation was also inhibited by the same less sensitive analysis of the metabolite than the parent drug (five times less), and their observation cannot be used as a general guideline since the study was performed on pigs and only involved four observations. Furthermore, controlled studied of single dose intake in humans such as the study on ketamine by Xiang et al. [13] would be interesting to perform with triazolam in order to obtain more knowledge on levels in hair and the influence of sweat and sebum on segments.

Fig. 1 Ion chromatograms (IS trace (D_5-diazepam), qualitative, and ▶ quantitative traces of triazolam) of extracts from **a** blank hair, **b** low standard at 0.3 pg/mg triazolam in hair and **c** case with1.0 pg/mg triazolam in hair


Std 0 Blind + IS
20100713-07JL Sm (Mn, 2x3)
a)
MRM of 11 Channels ES+
290.1 > 198.2 (Diazepam D5)
4.95e5
Area
20100713-07JL Sm (Mn, 2x3)
MRM of 11 Channels ES+
343 > 239.1 (Triazolam)
171
Area
20100713-07JL Sm (Mn, 2x3)
MRM of 11 Channels ES+
343 > 308.15 (Triazolam)
208
Area
Time
Std 1 0.0003 ng/mg + IS
20100713-08JL Sm (Mn, 2x3)
b)
MRM of 11 Channels ES+
290.1 > 198.2 (Diazepam D5)
8.76e5
Area
20100713-08JL Sm (Mn, 2x3)
MRM of 11 Channels ES+
343 > 239.1 (Triazolam)
900
Area
20100713-08JL Sm (Mn, 2x3)
9.61
98
MRM of 11 Channels ES+
343 > 308.15
1.48e3
Area
Time
RK20100977 Seg. 1 A vasket Tot 1
20100713-20JL Sm (Mn, 2x3)
c)
MRM of 11 Channels ES+
290.1 > 198.2 (Diazepam D5)
5.52e5
Area
0.40
186
0.91
257
20100713-20JL Sm (Mn, 2x3)
9.62
207
MRM of 11 Channels ES+
343 > 239.1 (Triazolam)
3.01e3
Area
20100713-20JL Sm (Mn, 2x3)
9.62
230
MRM of 11 Channels ES+
343 > 308.15 (Triazolam)
3.39e3
Area
Time


Blind + IS
20100901-16 Sm (Mn, 2x3)
a)
2: MRM of 5 Channels
347.06 > 312.15 (Triazolam
5.55e5
Area
20100901-16 Sm (Mn, 2x3)
2: MRM of 5 Channels ES+
359.1 > 176 (metabolite of triazolam)
451
Area
20100901-16 Sm (Mn, 2x3)
2: MRM of 5 Channels
359.1 > 141.05 (metabolite of triazolam)
1.26e3
Area
Time
Udr.std 0.002mg/L + IS
20100901-08 Sm (Mn, 2x3)
b)
2: MRM of 5 Channels ES+
347.06 > 312.15 (Triazolam D4)
6.43e5
Area
20100901-08 Sm (Mn, 2x3)
8.47
540
2: MRM of 5 Channels ES+
359.1 > 176 (metabolite of triatzolam)
7.84e3
Area
20100901-08 Sm (Mn, 2x3)
8.47
218
2: MRM of 5 Channels ES+
359.1 > 141.05 (metabolite of triazolam)
3.06e3
Area
Time
RK20101177 A urin
20100901-20 Sm (Mn, 2x3)
c)
2: MRM of 5 Channels ES+
347.06 > 312.15 (Triazolam D4)
5.14e5
Area
20100901-20 Sm (Mn, 2x3)
8.47
11274
2: MRM of 5 Channels ES+
359.1 > 176 (metabolite of triazolam)
1.64e5
Area
20100901-20 Sm (Mn, 2x3)
8.47
4729
2: MRM of 5 Channels ES+
359.1 > 141.05 (metabolite of triazolam)
6.92e4
Area
Time

◀ **Fig. 2** Ion chromatograms (IS trace (D_4-triazolam), qualitative, and quantitative traces of triazolam metabolite) of extracts from **a** blank urine, **b** low standard at 2 μg/l α-hydroxytriazolam in urine and **c** case with 39 μg/l α-hydroxytriazolam in urine

The detection of α-hydroxytriazolam in urine concurs with other studies of triazolam metabolism [14, 15]. These have reported that α-hydroxytriazolam is the major metabolite excreted in urine within the first 12 h. However, they also observed low amounts of it 30 to 40 h after intake. α-Hydroxytriazolam was present in amounts approximately 20 times greater than the second major metabolite 4-hydroxytriazolam [12]. The measured level of α-hydroxytriazolam in this case was comparable to the measurements of Tsujikawa et al. [14], who reported ranges from 8 to 800 μg/l urine.

This case presents data on the concentration of triazolam in human hair after a single or few doses. It was possible to corroborate the findings of the hair analysis by detection of a metabolite of triazolam in a urine sample taken from the assaulted person 20 h after the attack. The case illustrates that hair is a valuable forensic specimen in situations where natural processes have eliminated the drug from typical biological specimens due to delays in discovering the crime. Furthermore, hair segmentation allowed for a chronological interpretation of the analytical findings.

Acknowledgements The skilled laboratory assistance from Jytte Lundsby Jensen and Annemette Munch is gratefully acknowledged.

Conflict of interest The authors declare that they have no conflict of interest.

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