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Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines

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ABSTRACT

In China, benzodiazepines are the most frequently observed compounds in cases of drug-facilitated crime. Sensitive, specific, and reproducible methods for the quantitative determination of 18 benzodiazepines in hair have been developed using LC–MS/MS. Fourteen volunteers had ingested a single 1–6 mg estazolam tablet. Hair was collected 1 month after administration. All the proximal segments were positive for estazolam. With increased dosage, estazolam can be detected in the 2–4 cm segments in some subject's hair. Even some of 4–6 cm segments were positive. Hair analysis was applied to two authentic criminal cases. Full-length hair samples collected 5 weeks after the offense were cut into segments of 2 cm from the root, analyzed and quantified. The clonazepam concentrations measured in the first two segments for V#1 and V#2 were 15.47 and 11.93 pg/mg, respectively. However, both the 4–6 cm and the 6–8 cm segment of V1# remained positive, while those of V#2 were negative. It needs more substantial guidelines to use segmental hair analysis in drug-facilitated crime.

1. Introduction

Drug-facilitated crime (DFC) is a big problem in China. The majority of the cases are robberies. In addition, drug-facilitated sexual assaults (DFSA) have been increasingly reported. In China, benzodiazepines are the most frequently observed compounds in cases of drug-facilitated crime [1–4].

Benzodiazepines are the most widely and frequently prescribed sedative and hypnotic drugs worldwide and are, therefore, readily available. Their main pharmacological actions are hypnotic, antianxiety, muscle relaxant, and anticonvulsant effects. Benzodiazepines may also induce anterograde amnesia at therapeutic doses, with the risk increasing at higher dosages [5].

Blood and urine are the conventional specimens for documenting drug exposures [6–8]. In most cases, because of amnesia caused by drugs, there will be a 24–72 h or longer delay between a victim's report and the drug's ingestion. In addition, drugs used can be difficult to detect because low does were administered, or the active metabolite is chemically unstable. Some drugs are quickly cleared from the body fluids [9]. Therefore, in such circumstances, blood, and even urine samples are often of limited usefulness in detecting drugs' presence. To prolong the window of detection, hair analysis has been proven to be a solution. Actually, many publications on DFSA cases have demonstrated the usefulness of hair analysis in documenting the involvement of drug(s)/poison(s) [9–16].

Major progress achieved in the detection of benzodiazepines or hypnotics in hair following a single dose is a result of applying liquid chromatography (LC) coupled to MS/MS in forensic laboratories [17]. Kintz and coworkers have published a series of papers, including a general screening procedure [18] and specific methods for bromazepam [13,19], zolpidem [9], zopiclone [20] and alprazolam [15]. They recommended to cut the strand into three segments of 2 cm in order to document any drug-facilitated sexual assault case. Administration of a single dose would be confirmed by the presence of the drug in the proximal segment (root), with no detection in the other segments [17].

In the framework of the setup of a "segmental analysis" procedure based on the recommendations, our purpose was to devise a validated LC–MS/MS method for confirmation and quantification of 18 benzodiazepines in hair, and to apply the developed method to determine the levels of benzodiazepine in hair segments of (i) healthy volunteers after a single intake of estazolam and (ii) victims of two actual crimes.

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2. Materials and methods

2.1. Chemicals and reagents

Alprazolam, α-hydroxyalprazolam, midazolam, α-hydroxymidazolam, triazolam, α-hydroxytriazolam, estazolam, diazepam, nordiazepam, temazepam, oxazepam, clonazepam, 7-aminoclonazepam, flunitrazepam,7-aminoflunitrazepam, nitrazepam, 7-amnionitrazepam, flurazepam and diazepam-d5 were purchased from Cerilliant (Round Rock, TX, USA) and the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile, methanol, ammonium acetate, and formic acid were obtained from Fluka Chemical Co. (Buchs, Switzerland). Other reagents were all of analytical-reagent grade and no further purification was undertaken. Deionized water was purified using a Milli-Q system (Millipore, MA, USA).

2.2. Sample collection

2.2.1. Healthy volunteers

Fourteen healthy volunteers (ages 23–27 years) were recruited into the study. Subjects agreed to participate in the experimental part of the study through oral informed consent. All protocols were approved by an institutional review committee. Some background data were collected from the subjects such as the frequency of washing hair and the type(s) of hair cosmetic products used. Most subjects have straight black hair except for one female who has curled hair.

Blood samples were obtained before and at 1–8 h after a single oral dose of estazolam tablets (1 mg/tablet) with doses ranged from 1 to 6 mg were used in the study.

Hair was collected 1 month after administration. Strands of about 100 hairs were cut from the posterior vertex as close as possible to the scalp, oriented, and stored in clean paper bag at room temperature.

2.2.2. DFSA case

Two girls (V#1 and V#2) were invited to have a night time snack. After drinking a soft drink, they were unconscious. When they woke up, they realized that they had been raped and went to the police 18 h later. At the medicolegal unit of the hospital, blood samples were collected that revealed the presence of clonazepam and its metabolite 7-aminoclonazepam, confirming their previous declarations. When confronted with evidence of positive blood results, the suspects confessed. This was one criminal gangs case. Hair samples were collected 5 weeks after the offense.

Table 1
LC mobile phase gradient composition.

LC run time (min)	Acetonitrile (%)	Ammonium acetate buffer (%)[a]
0	5	95
3	60	40
7	80	20
20	80	20
20.5	5	95
30	5	95

[a] 20 mM ammonium acetate buffer with 0.1% formic acid, pH 4.0.

2.3. Sample preparation

2.3.1. Hair samples

Hair was segmented and rinsed twice with 5 ml dichloromethane. The last wash was stored for further analysis. After being air-dried, the segments were cut into small pieces of less than 3 mm, and pulverized in a freeze mill (Freezer/Mill, SPEX CertiPrep). 20 mg of powered hair was sonicated in an ultrasonic bath in 1 ml of phosphate buffer, pH 8.4, for 1 h at room temperature, in the presence of 0.4 ng diazepam-d5 as internal standard (IS). Liquid–liquid extraction was performed with 3 ml of dichloromethane. After solvent evaporation at 60 °C, the residue was reconstituted with 100 μl of acetonitrile–20 mM ammonium acetate (70:30, v/v), and 5 μl was injected into the LC–MS/MS system.

2.3.2. Blood samples

The extraction procedure was the same as that previously reported [21]. To 1 ml of blood sample, 10 ng diazepam-d5 (IS) and 2 ml of sodium borate buffer, pH 9.2, were added. Liquid–liquid extraction was performed with 3 ml of diethyl ether.

Table 2
MRM transitions, condition and retention time for benzodiazepines.

Compound	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	Dwell time (ms)	DP (V)	CE (eV)	Rt (min)
Diazepam	285.1	193.3	30	60	45	9.91
		154.1	30		36	
Oxazepam	287.2	241.2	30	50	31	8.55
		269.3	30		36	
Nordiazepam	271.2	140.2	30	60	36	9.14
		208.1	30		36	
Temazepam	301.2	255.2	30	70	36	9.25
		283.1	30		19	
Clonazepam	316.2	270.1	30	65	36	9.11
		214.1	30		49	
7-Aminoclonazepam	286.1	222.2	30	60	34	7.73
		250.1	30		42	
Nitrazepam	282.2	236.2	30	60	32	8.95
		180.2	30		52	
7-Aminonitrazepam	252.2	121.1	30	80	37	7.69
		146.2	30		38	
Flunitrazepam	314.2	268.3	30	65	35	9.62
		239.3	30		45	
7-Aminoflunitrazepam	284.2	135.2	30	80	39	8.08
		226.2	30		41	
Triazolam	343.2	308.2	30	70	36	8.93
		315.2	30		35	
α-Hydroxytriazolam	359.2	331.2	30	80	38	8.23
		176.1	30		37	
Alprazolam	309.2	281.1	30	70	32	8.94
		274.2	30		33	
α-Hydroxyalprazolam	325.2	297.2	30	70	35	8.23
		279.2	30		33	
Midazolam	326.2	291.4	30	70	37	11.65
		244.2	30		35	
α-Hydroxymidazolam	342.0	324.2	30	60	29	9.26
		203	30		38	
Estazolam	295.2	267.3	30	70	34	8.67
		205.2	30		53	
Flurazepam	388.2	315.2	30	55	32	19.66
		288.1	30		33	
Diazepam-d5 (IS)	290.2	198.2	30	60	45	9.91
		159.2	30		36	