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Case Report
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Alprazolam and Zolpidem in Skeletal Tissue of Decomposed Body Confirms Exposure

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Abstract

In several medico legal cases bone samples analysis may provide the only source of toxicological information. The present case study reports the analysis of a segment from a human bone, belonging to a 46-year-old man 3 months after his death. Bone was the only available material for toxicological analysis, among few skull hair and rotten skin. Analysis was performed by UPLC-MS/MS following simple and efficient sample pretreatment. The results were in accordance with the man's medical record: alprazolam and zolpidem were found at 2.2 and at 5.4 ng/g of bone respectively. Both these drugs were prescribed to the deceased.

Keywords: Skeletal tissue; Drug exposure; Alprazolam; Zolpidem; Femur

Introduction

During autopsy, apart from common biological fluids such as blood and urine, alternative samples like tissues, hair or bones can be collected for toxicological testing. All these samples aim to provide useful information about the cause of death. In cases where the body has undergone significant decomposition, skeletonization, exsanguinations or fragmentation [1] the analysis of skeletal tissue may provide the only source of toxicological information. Thus when a deeper medico-legal investigation is needed in such cases, bone's analysis can provide critical information and therefore the development of appropriate methods for the detection of drugs and pharmaceuticals in human bones is important. Skeletal tissue may provide useful information for the determination of drug exposure. However, no safe conclusions can be extracted by bone or bone marrow analysis that would give clear information about dosing, frequency and length of time since last drug use [2]. In the present case study, a part of thigh bone was analyzed by an UPLC-MS/MS method developed in our laboratory. The sample pretreatment is simple and the developed UPLC-MS/MS method is effective for the drug analysis of human bone samples. The overall aim of the study was to apply an alternative aspect of toxicological analysis in a decomposed and almost skeletonized human body.

Case Report

A 46-year-old man, with psychiatric history, leaving in a village at the area of Northern Greece, left his home on the 4th August 2016 riding his motorbike and from then his signs was missing. There were no evidences explaining his disappearance. (According to his family there were no reasons for leaving in such a way). His disappearance was reported to the local police department the same day by his parents. His family and friends went searching the area and the nearby forest with no result. The man was facing significant psychiatric problems and had a medical history of drug abuse. Specifically, STILNOX (zolpidem) and XANAX (alprazolam) prescribed to him. Three months after the report of his disappearance, on the 2nd of November 2016, the body of the 46-year-old male motorcycle driver was found in the surroundings. The deceased was found "sitting" on his bike with the vehicle lying on the right, in close distance from the main road, out of town. His right leg was found under the bike. The bike and the driver were covered by bushes, leaves and grass. A helmet was found in the bike top box (Figure 1). The body was found decomposed in skeletonised state, with his clothes on and personal belongings in his pockets and on the bike (identity card, keys, cigarettes e.t.c.). Among them two vials of the drug Xanax (alprazolam) were found. Initially, the case was registered as road traffic accident by the police and the body was shifted to a mortuary for autopsy.

Autopsy Findings

The forensic pathologist reported a decomposed male body, 170-180 cm long, with mummifying

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Figure 1: The deceases (black human sketch) and the motorbike (white circle) as found in the bushes from the Police Authorities.

Figure 2: The part of bone (red box) which was taken for further toxicological analysis.

tissues, insects and worms on the surface and in body cavities, with summer clothing on. On further examination the following observations were recorded: decapitation of the cranium occurred as result of the accident. Facial and cranial bones were found intact. No remaining brain tissue could be found. Multiple fractures of the cervical spine and dried blood hematoma were observed. In addition, thoracic injury on the right and fracture of right ribs from 3rd to 8th at sternocostal junction were observed. Remaining dried tissues in the thoracic and intraperitoneal region, intact thoracic and lumbar spine and dried haematoma on the remaining aortic tissues were found. The cause of death as reported from the forensic pathologist was: cervical – thoracic injuries compatible with traffic accident. Upon autopsy a small piece of sample was taken from the left thigh bone, shown in (Figure 2), for toxicological analysis.

Materials and Methods

Chromatography was performed by an ACQUITY UPLC H-Class System – (Waters Corporation) on an Acquity BEH C18 column (150 × 2.1 mm i.d., 1.7 µm; Waters) protected by an Acquity BEH C18 Van Guard pre-column (5 mm × 2.1 mm i.d., 1.7 µm; Waters) using a mobile phase of A: water, 0.1% formic acid over B: methanol, 0.1% formic acid. MS/MS detection of analytes and IS was carried out on a Xevo TQD system (Waters, UK) with electrospray ionization operating in positive and in negative mode on switching mode. Cone voltage and collision energy were optimized for each transition. Data acquisition and analysis was performed by MassLynx® software whereas quantitation was performed by TargetLynx application.

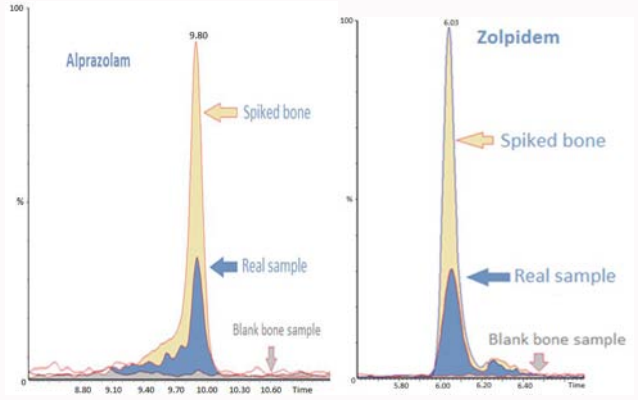

Figure 3: Chromatographic traces of the detected drugs in blank (grey) bone, spiked (yellow) and case (blue) sample.

Table 1: A list of the 27 target analytes determined by the applied UHPLC-MS/MS method.

Compound	
EME	Zolpidem
Morphine	Venlafaxine
Codeine	Clozapine
Olanzapine	Citalopram
6-MAM	Biperiden
MDA	Bromazepam
Amphetamine	Methadone
MDMA	Amitriptyline
Methamphetamine	Alprazolam
MDEA	Nor-diazepam
BE	Diazepam
Mirtazapine	THC-COOH
Cocaine	D9-THC
7-AF	

All chemicals used were of LC-MS analytical grade. Methanol LC-MS grade was purchased from Fisher Scientific International Inc. (Hampton, NH, USA) and formic acid (>98%) was purchased from Riedel-de Haën® (Sigma-Aldrich, Steinheim Germany). HPLC grade water was obtained by Milli-Q purification system. Compounds of the highest available purity were used as standards and internal standards were supplied by Lipomed (Arlesheim, Switzerland) and Cerilliant (Texas, USA). The bone sample that was chosen for analysis was from femur; a small piece was collected and weighted. The sample was analyzed by an UHLC-MS/MS method which was developed for the determination of 27 drugs and pharmaceuticals. The target compounds are listed in (Table 1). The identification of the drugs was performed by the retention time and by two SRM transitions in the mass spectrometer.

Sample Preparation

The bone sample was washed with deionized water until the washings were clear and the bone clean without external contamination (soil etc.). After the bone sample was dried in the air, any remains of muscles or other kind of tissues were removed meticulously using a scalpel. The bone segment was then grounded using a mortar. From the pulverized sample 1 g was weighed in a vial.

In this 3 mL of MeOH and 12.5μL of NH_4OH (13.4 M) were added (pH 10) and the mixture was left in an orbital shaker for 5 hours. Ultrasonication for 1 hour, centrifugation at 10.000 x g for 10 min and filtration (0.22μm) followed. The clear extract was then evaporated to dryness under a nitrogen stream and the residue was reconstituted with 300 μL of a mixture H_2O: MeOH – 80:20 v/v, filtrated (0.22μm) again and transferred to an auto sampler vial for analysis. For the determination of drugs 1 g of a blank bone sample and 1 g of the same blank bone sample, spiked with a mixture of the 27 drugs were treated in the same way as described above and analyzed together with the case sample. The blank bone sample was collected from a man with no history of prescribed medication and with negative results after the analysis of his post mortem blood and urine.

Results and Discussion

In the femur sample analyzed using the developed UPLC-MS-MS method two pharmaceutical drugs were detected and quantified. More specifically, alprazolam and zolpidem were found at concentrations of 2.2 ng/g and 5.4 ng/g respectively. These findings are in agreement with the known drug history of the deceased. Chromatograms obtained by a blank bone sample, a spiked bone sample with alprazolam and zolpidem and the examined case bone specimen are shown in (Figure 4). These findings have forensic importance especially in cases with skeletonized remains, as we can have a drug history of the deceased. Relative available literature data are limited to conclude any direct or indirect involvement of the drugs in the cause of death of the deceased. One safe conclusion is that alprazolam and zolpidem can be detected in bones three months after the death. Questions that have to be answered in relation to bones and drugs, thus to extract safe conclusions from analysis of post-mortem specimens, are at least: what are the factors determining the deposition of a drug in bones? Which is the correlation between drug bone concentration and the corresponding concentration in blood? What is the stability of a drug in post-mortem bones under burial or different environmental conditions? The literature is quite limited but gives some information. According to the current literature, there have been cases where drugs were found in much higher concentrations in bone than in the postmortem blood. Characteristic examples are desipramine, flurazepam and pentobarbital which were found in about thirty times higher than in blood [3-5]. However, there are reports in the literature of drugs, such as olanzapine, that have not been detected in bones even if they were present in blood [6]. In a study performed in mid femoral bone specimens for thirteen drugs (amitriptyline, doxepin, dothiepin, mianserin, moclobemide, sertraline, chlorpromazine, thioridazine, clozapine, diazepam, oxazepam, and temazepam), out of the 29 cases which were blood positive in these drugs, 25 cases were also positive in their bone samples. Apart from that the majority of these drugs and their primary metabolites were found in bone tissue, their concentrations also matched the confirmed blood results, in both therapeutic and classic overdose ranges [7]. It has been proven that bones may preserve different kinds of compounds for long period of time as there are several case reports in which skeletal remains found positive in drugs years after death, such as methamphetamine and amphetamine which were detected 5 years after death in dry marrow [8]. Another example is triazolam which has also been detected in bone marrow of a victim 4 years after death [9]. Data have also been reported for morphine where a loss of 54.4% of morphine concentration was occurred after 1-year burial of post-mortem bones of a fatally poisoned heroin victim [10]. Amitriptyline, nortriptyline, citalopram, and desmethylcitalopram distribution was examined in 13 different porcine bones after acute exposure and outdoor decomposition for 2 years. The authors concluded that the bone type was the main effect with respect to drug level of the analytes examined, with levels varying from 33- to 166-fold [11]. With respect to benzodiazepines, it has been found that they have limited stability in bone structures over long periods of time (months) [12]. The found concentration of alprazolam and zolpidem in the present case can give limited information based on the existing literature. The instability of benzodiazepines in bone matrix poses limitations in the interpretation of alprazolam level in bone and there are no previous data on zolpidem. Our findings however confirmed the drug history of the deceased and the ability of detection of alprazolam and zolpidem in bones three months after death.

Conclusion

In conclusion, further studies should be done to elucidate the correlation between post-mortem bone and blood concentrations, the stability over time of various drugs in different burial and environmental conditions and the role of bone tissue as repository or not for drugs. Finally, even if it is not realistic at this time to estimate the level or duration of exposure, the detection of a drug in post-mortem bones may be at least a proof of the deceased's exposure to this substance [13].

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