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UPLC-MS/MS Method for the Determination of Drugs of Abuse and Pharmaceuticals in Skeletal Tissue

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APPLICATION BENEFITS

- Bone analysis as a useful tool of forensic investigation, especially in cases where other specimens are unavailable.
- Bone analysis can provide useful and reliable data for medico-legal death investigations.

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Xevo™ TQD Mass Spectrometer

ACQUITY UPLC BEH Column

VanGuard™ Column Protection

KEYWORDS

UPLC-MS/MS, bone, skeletal, medico-legal, postmortem

OBJECTIVE

Development of a simple method for the detection and quantification of 27 drugs and pharmaceuticals in human bones. The target compounds comprise pharmaceuticals (antipsychotics and antidepressants) and some of the most important groups of drugs of abuse: opiates, cocaine, cannabinoids, amphetamines, and benzodiazepines.

INTRODUCTION

In terms of medico-legal death investigations, apart from common specimens such as blood and urine, alternative samples like tissues, hair, or bones are collected at autopsy for toxicological testing. Each specimen provides useful, complementary information in such investigations. In cases where the body has undergone significant decomposition, skeletonization, exsanguination, or fragmentation, the analysis of bone may provide the only source of toxicological information.[1] Therefore, there is an important need for the development of appropriate methods for the detection of drugs and pharmaceuticals in human bones in medico-legal cases, such as the investigations into the cause of death. A method has been developed for the unambiguous detection and sensitive determination of drugs of forensic interest, with a simple sample preparation protocol.

MATERIALS

BONE SAMPLES

The samples were collected during autopsy, 14–20 hours postmortem, where a small piece of the right femur was taken. All of them belonged to males with confirmed history of taking pharmaceuticals and illicit drugs.

REFERENCE STANDARDS

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). Cocaine-D3 was used as an internal standard (IS) at a concentration of 100 ng/g of bone.

CALIBRATORS

Individual stock solutions for the compounds of interest were prepared at 1 mg/mL in methanol. Mixed working solutions, for the preparation of bone calibrators, were prepared by further dilution with methanol to yield mixed spiking solutions at 0.1, 1, 10, and 100 µg/mL in methanol. All stock solutions and dilutions were stored at -20 °C. A series of bone calibrators ranging from 5–5000 ng/g bone were prepared by spiking drug-free human bone with mixed spiking solutions.

EXPERIMENTAL

Sample preparation

Each bone was washed with deionized water until the water fraction was clear and the bone was left clean without external contamination (e.g., soil, etc.), then the bone was left to dry in the air. After that, any remaining muscles or other types of tissues were removed meticulously using a scalpel. Afterwards the bones were ground using a mortar. The crushed bones were stored at -40 °C until analysis.

In terms of analysis, frozen bone samples were initially placed on the bench to reach room temperature before taking 1 g of bone together with 3 mL of methanol. The pH of the mixture was adjusted to pH 10 by the addition of 12.5 µL of NH_4OH (13.4 M). The bone mixture was placed on a rotary mixer for five hours before placing in an ultrasonic bath for a further hour. The sample was centrifuged at 10.000x g for 10 min and, after filtration (0.22 µm) to remove bone remains, the supernatant was transferred to another bottle. The supernatant was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 300 µL (water:methanol, 80:20 v/v), filtered (0.22 µm) again, and thereafter transferred into an autosampler vial for analysis.

LC conditions

LC system:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC BEH C_{18} (150 mm x 2.1 mm I.D., 1.7 µm) protected by an ACQUITY UPLC BEH C_{18} VanGuard Pre-column (5 mm x 2.1 mm I.D., 1.7 µm)
Column temp.:	50 °C
Mobile phase A:	Water containing 0.1% formic acid
Mobile phase B:	Methanol containing 0.1% formic acid
Wash solvent:	Methanol:acetonitrile:water containing 0.1% formic acid (70:20:10, v/v)
Purge solvent:	Water:methanol (80:20 v/v)
Injection volume:	5 µL
Gradient elution:	(Table 1)

MS conditions

MS system:	Xevo TQD
Data acquisition and processing:	MassLynx™ with TargetLynx™
Ionization mode:	ESI+/-
Capillary voltage:	ESI + 3.5kV, ESI - 2.5kV
Acquisition mode:	Multiple reaction monitoring (MRM – Table 2)

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0	0.300	80.0	20.0	Initial
2.00	0.300	80.0	20.0	6
2.13	0.300	68.1	31.9	6
6.80	0.300	66.9	33.1	6
6.81	0.300	30.0	70.0	1
10.00	0.300	1.0	99.0	6
13.00	0.300	1.0	99.0	1
17.00	0.300	80.0	20.0	1

Table 1. Gradient conditions, total run time of 17.0 min.

Compound	Rt (min)	Precursor ion (Q1)	Product ions (Q3)	Cone voltage (V)	Collision energy (eV)	Ionization mode
Ecgonine methyl ester	1.17	200	82*/182	30	18	+
Morphine	1.28	286	185*/201/173	60	25	+
Codeine	1.80	300	165*/215	18	44	+
Olanzapine	2.23	313	84*/256	46	24	+
6-MAM	2.24	328	193*/211	50	25	+
MDA	2.92	180	163*/135/133	18	10	+
Amphetamine	2.94	136	91*/119	20	18	+
MDMA	3.01	194	163*/135/105	25	12	+
Methamphetamine	3.09	150	91*/119	28	15	+
MDEA	3.77	208	163*/72/133	25	12	+
Benzoylecgonine (BE)	4.67	290	168*/105	35	18	+
Mirtazapine	5.17	266	72*/195	42	28	+
Cocaine	5.26	304	82*/182	40	20	+
7-Aminoflunitrazepam	5.48	284	135*/227	52	32	+
Zolpidem	6.05	308	92*/235	50	35	+
Venlafaxine	8.81	278	58*/260	28	20	+
Clozapine	9.04	327	84*/270	42	22	+
Citalopram	9.10	325	109*/262	45	30	+
Biperiden	9.33	312	98*/143	38	23	+
Bromazepam	9.35	316	209*/182	46	40	+
Methadone	9.40	310	105*/265	30	14	+
Amitriptyline	9.44	278	91*/104	45	30	+
Alprazolam	9.79	309	281*/274	40	25	+
Nordazepam	9.97	271	165*/140	50	25	+
Diazepam	10.14	285	154*/193	58	28	+
THC-COOH	11.39	343	299*/325	50	22	-
Delta-9-THC	11.44	313	245*/191	50	25	-

Table 2. Analytical parameters used for MRM monitoring.

** The most abundant ion was used for analyte quantification.*

RESULTS AND DISCUSSION

The scope of this work was the development of a sensitive, specific, but also robust analytical tool that would enable the analyst to provide reliable data from "difficult" samples and quantify trace amounts of drugs. Due to the nature of the samples and the importance of the anticipated result, detection and identification of drugs should be reliable. Finally, analysis should not be time-consuming and should not cause system degradation, such as column or source capillary blockage. It can be understood that meeting such requirements necessitates the optimization of both the LC and the MS method.

The method was fully validated, and the results are summarized below (full details are available in the manuscript).[1]

LINEARITY

Calibration curves were constructed at seven concentration levels (5, 50, 100, 500, 1000, 2000, and 5000 ng/g in bone) and three replicates performed for each calibrator. Regression lines were assessed based on peak area ratios of each of the 27 compounds to that of the internal standard. The method showed dynamic linear range r^2 >0.9907. The limit of detection (LOD), based on an S/N of 3/1, was between 0.03 and 1.35 ng/g depending on the analyte. The limit of quantification (LOQ) was based on 3.3x the LOD, and ranged from 0.11 to 4.15 ng/g.

ACCURACY, PRECISION, AND SELECTIVITY/SPECIFICITY

The method showed good precision. Intraday precision, expressed as %RSD, was below 10% in most cases with the highest value at 11.92%. Interday precision was again below 10% in most cases, with the highest %RSD value at 13.86%, indicating satisfactory repeatability.

The specificity of the method was satisfactory. All chromatographic peaks were well separated, and no cross talk was observed (Figure 1). In addition, no peaks from endogenous substances interfered or coeluted with the analytes in the monitored transitions. Furthermore, analysis of blank bone samples from 20 subjects showed no interfering peaks with the analytes of interest.

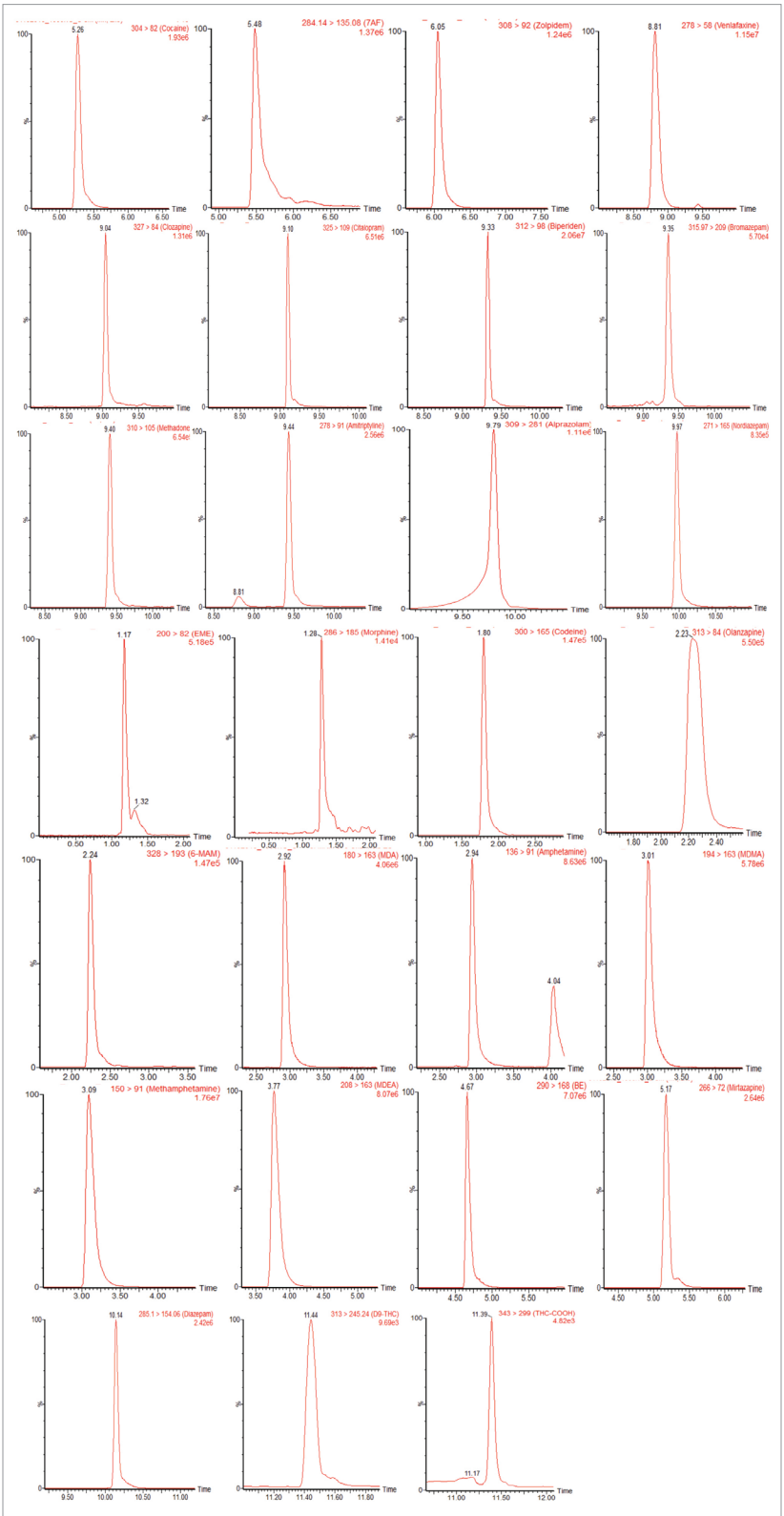

Figure 1. Chromatographic peaks of 27 drugs found in skeletal tissue.

Accuracy was calculated from the calibration curve at three quality control concentrations, i.e., low QC (50 ng/g), medium QC (500 ng/g), and high QC (5000 ng/g). Results indicated acceptable accuracy ranging between 82.1% to 113.6%.

MATRIX EFFECT AND CARRY OVER

Average matrix effects, evaluated at 50 ng/g and 500 ng/g, were 11% and 16% suppression, respectively. The highest matrix effects were found for benzoylecgonine (~30% suppression). Carryover was less than 1% in the blank extracted bone samples when analyzed after an injection of 5000 ng/g.

RECOVERY

Recovery of the analytes, assessed by the ratio of responses of spiked bone prior to and post extraction, at 50 ng/g and 500 ng/g, ranged from 80.4% to 116.5%.

STABILITY

Short-term stability was assessed in working solutions and extracted bone samples at 100 ng/g. Samples were found to be stable for at least 48 hours in the autosampler (temperature maintained at 4 °C) as well as at -20 °C for up to 72 hours.

APPLICATION TO REAL SAMPLES

The method was applied to bone samples collected from six forensic cases.

The first two cases were postmortem samples from chronic drug abusers. Analysis of fresh thigh bone samples from these individuals resulted in the quantification of eight drugs: five in case A (cocaine and its metabolite benzoylecgonine, morphine, codeine, and bromazepam) and three in case B (cocaine, benzoylecgonine, and codeine).

To further test the capability of the method, burial conditions were simulated, and the samples were re-analyzed after a burial period of one year. All drugs showed a decrease in concentration; in case A, cocaine and benzoylecgonine were still detected in bone and bone marrow, but concentrations were below the LOQ. Morphine and codeine could be quantified but concentrations were decreased by ~90%. Bromazepam was only detected in bone marrow; concentrations were ~50% of those in fresh bone.

For case B, no drugs were detected in bone after the one-year burial period, but bromazepam and cocaine were detected in bone marrow, again at decreased (~50%) concentrations in comparison with fresh bone.

In another study, the method was also applied to a historical collection of four bone samples, which had been buried for a period of one year. Detected drugs included opiates, cocaine and metabolite, benzodiazepines and metabolites, and antidepressants.

CONCLUSIONS

LC-MS/MS analysis of bone offers potential for the unambiguous detection and quantitative analysis of pharmaceuticals and drugs of abuse at low concentrations. The optimization of the extraction method led to the development of an efficient and quick method (about six hours in duration), which is shorter than the overnight extraction typically cited in the literature. The method was validated extensively and subsequently applied to the analysis of samples from six forensic cases. In these samples, 34 drugs were successfully identified; of these, 27 could be quantified.

Importantly, 26 compounds were detected and 20 quantified in bone or bone marrow one year after burial. These results provide evidence to support bone analysis as a useful tool of forensic investigation, especially in cases where other specimens are missing. The most important conclusions to be derived is that forensic toxicological analysis can still be performed when common biological samples (blood, urine, tissues, or hair) are missing. Even when the only available sample is a part of a skeletonized human body, the described methods can provide reliable data to the legal medicine expert.

References

1. Orfanidis, A. et al. Determination of Drugs of Abuse and Pharmaceuticals in Skeletal Tissue by UHPLC-MS/MS. *Forensic Science International*, **2018**, 290: 137–145.

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