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Skeletal tissue, a viable option in forensic toxicology? A view into post mortem cases

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Highlights

- Comparison of drug concentrations in bone tissue, bone marrow and blood
- Bone (marrow) shows great potential for screening purposes in post mortem forensic toxicology
- A trend was seen between blood drug concentrations and bone (marrow) drug concentrations for methadone
- A trend was seen between blood drug concentrations and bone (marrow) drug concentrations for citalopram

Abstract

Blood analysis is the golden standard in the field of forensic toxicology. However, when extended decomposition of the remains has occurred, alternative matrices are required. Skeletal tissue may provide an appropriate sample of choice since it is very resistant to putrefaction. However, today, the absence of reference data of drug concentrations in skeletal tissue poses a problem to meaningfully and reliably conduct toxicological testing on human skeletal material. The present study investigates the viability of skeletal tissue as an alternative matrix to evaluate xenobiotic consumption in legal cases. Blood, bone tissue and bone marrow of different forensic cases were screened for 415 compounds of forensic interest. Afterwards, methadone, clomipramine, citalopram and their respectively metabolites positive samples were quantified using fully validated methods. Sample preparation was carried out by SPE (whole blood and bone marrow), methanol extraction (bone sections) or protein precipitation (whole blood). All samples were analyzed using liquid chromatography coupled to a triple quad mass spectrometer. Multiple drugs were successfully identified in all sampled matrices. In bone (marrow) not as many substances were detected as in blood but it poses a valid alternative when blood is not available. Especially bone marrow showed big potential with a concordance of 80.5% with blood. Clomipramine, citalopram and their metabolites were proven to be detectable and quantifiable in all specimens sampled. Bone marrow showed the highest concentrations followed by blood and bone tissue. When citalopram blood concentrations were correlated with the bone concentrations, a linear trend could be detected. The same was seen between blood and bone marrow for citalopram concentrations. Methadone was also proven to be detectable in all specimens sampled. However, its metabolites EMDP and EDPP were absent or below the LOD in some samples. Overall, methadone concentrations were higher in bone marrow than in bone. With exception of one case, blood concentrations were higher than bone concentrations. For methadone, a linear trend could be found between blood and bone concentration. Comparing methadone concentrations in blood and bone marrow an exponential trend could be seen. In conclusion, these findings show the potential forensic value of bone and bone marrow as an alternative matrix. Aside to that, a standard protocol for the sample collection and processing is proposed.

Keywords: skeletal tissue, post mortem

1. Introduction

In forensic toxicology, blood and urine are the golden standard for analysis^[1]. For these matrices, a lot of reference material is available. This makes it highly feasible for the unambiguous identification and quantitative determination of drugs. Sometimes however in cases where the body is extremely putrefied, these traditional matrices are unavailable so that alternatives are required^[2]. Although many alternative specimens are investigated over the past years to detect the presence of drugs or other toxicological substances, very little attention was given to the usage of skeletal tissue^[3]. When looking at previous case reports, drawing unambiguous conclusions about dosing or length of time since last drug use cannot be determined from skeletal tissue analysis. Although skeletal tissue has a number of advantages over other alternatives, it has been shown to be a depot for certain drugs^[4-6]. Skeletal tissue also withstands putrefaction best of all specimens. Skeletal tissue consists of two major categories being bone tissue and bone marrow^[7]. In the right conditions, bone tissue being hundreds of years old can be found^[8].

Several studies have already been performed on detection of drugs inside bone tissue^[4,6,9,10]. Within a single bone, the drug concentration shows a gradient. Parts of bone containing more trabecular bone tissue appear to have higher concentration compared to the more compact cortical bone tissue^[4]. This can be accounted for by the higher grade of vascularization in trabecular bone. Aside to the variance seen within a single bone, there is also a big variance seen in the drug concentration found within the different bones of the skeleton. Long bones tend to give higher concentrations compared to the small and irregular bones^[4]. This again can be explained by a difference in the vascularization rate. Although the drug concentration seems to be depended on vascularization, no significant correlation with blood concentrations could be found in animal experiments^[9].

On the inside of the bone, a different alternative matrix is found. The reddish gelatin substance is called bone marrow^[7]. Bone marrow has the benefit that it is well protected from any contamination as long as the bone is intact^[11]. This makes it very suitable in case of extreme decomposition. Its major function is the production of blood cells. It also plays a major role in providing the cells for bone formation^[7]. These functions are mainly performed by the red marrow (medulla ossium rubra). Aside to the red marrow, there is also yellow marrow present (medulla ossium flava). These are fat cells or adipocytes. The amount of yellow marrow increases during a lifetime. By the time of adulthood, it compromises 70% of the bone marrow mass. Its distribution is not equally across the body. Higher concentrations are found in the arms and legs. This fatty texture favors diffusion of the more lipophilic

substances from the blood to the bone marrow^[12]. Also for bone marrow, most studies that have been undertaken, use an animal model to investigate the influence of dosing and other parameters^[6,13–15]. Different categories of drugs are detected in bone marrow. A few studies compared the ability of detecting substances between bone marrow and blood but came to different conclusions depending on the analyte, dosing and interval between death and administration. The sensitivity of bone marrow to blood varied from 45% to 100%^[11,16]. The quantitative approach showed a small correlation for some xenobiotics between blood and bone marrow^[17]. However, a lot of discrepancy in results have been described^{[18][19]}. Various reasons for the discrepancies have been proposed going from different sampling locations to age and storage conditions^[11].

The interpretation of drug concentrations found in skeletal tissue remains very hard. To date, most studies have used animal models to investigate the influences of dosing conditions, administration routes and other factors on the drugs concentrations^[6,9,20,21]. This gave an idea about the mechanisms behind drug incorporation into skeletal tissue. However, at the moment, the absence of reference data of drug concentrations in human skeletal tissue poses a problem to conduct meaningfully and reliably toxicological testing on human skeletal material. The absence of reference data in humans triggered this research project. In this study, blood, bone marrow and bone drug concentrations of legal cases will be compared to each other. The first question that arises is: can all xenobiotics be detected in bone marrow and bone tissue? Since both are highly vascularized, one may assume so. So in a first step, these matrices will be screened using a commercially available screening method for 415 compounds of forensic interest. The efficacy to detect compounds will be compared to that of the more traditional matrix namely blood. The second question to address is: are drugs levels found in bone or bone marrow representative for blood levels at time of death? The literature does not give a clear answer to this question. Correlation between blood and BM was already demonstrated for multiple drugs^[16,17]. However, this data is based on animal experiments in a standardized setting. Recently, Vandenbosch et al. validated a methanolic extraction coupled to liquid chromatography—tandem mass spectrometry (LC-MS/MS) for the quantification of methadone, citalopram, clomipramine in bone tissue^[4,9]. From a forensic perspective, it is useful to evaluate and to apply the validated method for analysis on postmortem human samples to collect reference data. The aim of this study was to demonstrate the efficacy to detect drugs of forensic interest in bone (marrow) and the

suitability of bone (marrow) as a valid specimen for toxicological analysis in cases of extreme decomposition and exhumation.

2. Materials and methods

In this project, the ability to detect drugs of forensic interest in bone (marrow) and the suitability of bone (marrow) as a valid specimen for toxicological analysis was evaluated. In a first stage, these specimens were compared regarding their ability to detect drugs in a standard routine screening. In a second stage, validated methods were applied to these specimens to quantify citalopram, clomipramine, methadone and their metabolites.

2.1. Chemicals and reagent

Analytical reference standards of clomipramine (1mg/mL), clomipramine.d3 (100 µg/mL), desmethylclomipramine (1mg/mL), citalopram (1mg/mL), citalopram.d6 (100 µg/mL), desmethylcitalopram (1mg/mL), desmethylcitalopram.d3 (100 µg/mL), methadone (1mg/mL), methadone.d3 (100 µg/mL) EDDP(1mg/mL), EDDP.d3 (100 µg/mL) and EMDP (1mg/mL) were purchased from LGC standards (Teddington, UK). Methanolic standard stocks of different concentrations were prepared by mixing reference standards. Separate methanolic standard stock solutions of deuterated analogues were prepared. All standard solutions were stored at -20 °C.

All solvents, chemicals and reference standards were at least of analytical or HPLC grade. Acetonitrile and methanol were obtained from Biosolve (Valkenswaard, The Netherlands). Dichloromethane, acetic acid, 2-propanol, monopotassium phosphate and ammonium hydroxide which were used in the sample preparation were purchased from Merck (Darmstadt, Germany).

Formic acid and ammonium formate were purchased from Sigma-Aldrich (Bornem, Belgium). Deionized water was prepared using a Milli-Q Water Purification System (Millipore, Brussels, Belgium). The aqueous buffer for method A and B was prepared as followed: 10mM of ammonium formate, adjusted to pH 4 with formic acid. For the screening method, the aqueous buffer was prepared as followed: 2 mM of ammonium formate, adjusted to pH 2.6 with formic acid.

Bond Elut Plexa PCX cartridges (60 mg, 3 ml) were purchased from Varian (Sint-Katelijne-Waver, Belgium). All solid phase extractions (SPE) were carried out on a Vac Elut SPS 24 (Varian, Sint-Katelijne-Waver, Belgium).

2.2. Sample collection

Human post mortem samples were obtained at autopsy of legal cases at UZ Leuven (Belgium) during the period from April 2018 to September 2019. For each case, the background and the medical history is reported as provided by the legal system. Cases were selected after a positive screening result for methadone, citalopram or clomipramine in blood using the method as described further on. The clavicle bone was chosen as specimen of choice due to the high accessibility during autopsy. After removal of the breastplate, a ring of 1 cm width was serrated 1 cm from the center of the proximal clavicle head. Figure 1 shows an example of a serrated clavicle bone. Bones were cleaned of soft tissue with a scalpel and with a sanding machine. This ring of bone was placed on a self-made construction in a testing tube. The construction is shown in suppl. figure 1. This testing tube was centrifuged for 15min at 3500 rpm till all bone marrow was collected at the bottom. 100 mg of bone marrow was taken for analysis. The inside of the ring was further cleaned off bone marrow traces using a sanding machine. A blood sample was also taken during autopsy. Approval for this study was received from the Medical Ethics Committee of the faculty of Medicine of the University Hospital of Leuven, Belgium.

2.3. Specimen preparation

2.4.

2.4.1. Blood and bone tissue

Blood samples were extracted in duplo. Once using a simple protein precipitation procedure as reported by our group ^[9,20]. The second time using an SPE method as described by our group ^[22]. Bone samples were extracted using a methanolic extraction as recently reported by our group ^[9,20].

2.4.2. Bone Marrow

In a glass tube, 100 mg bone marrow was mixed with 2 ml 0.1 M phosphate buffer pH 6 and 10 µL of clomipramine.d3 (10µg/mL), 10 µL of citalopram.d6 (10µg/mL), 10 µL of desmethylocitalopram.d3 (10µg/mL), 10 µL of methadone.d3 (10µg/mL) and 10 µL of EDDP.d3 (100ng/mL). The solution was sonicated for 15 min in an ice bath. The SPE cartridge was conditioned with 3 mL methanol, 3mL MiliQwater and 1 ml 0.1 M phosphate buffer pH 6. The bone marrow solution was vortexed and centrifuged for 10 min at 3500 rpm. This solution was cooled in an ice bath until the fat layer on top was frozen. While avoiding the fat layer, the supernatant was loaded on the SPE cartridge. The loaded cartridge was

washed with 3 mL MilliQwater, 1 mL 1 M acetic acid and finally 3 mL methanol. Afterwards the SPE column was dried for 10 minutes. The analytes were eluted with 3mL of a mixture containing 1:10:39 amonium-isopropanol-dichloromethane. The eluates were evaporated to dryness under a gentle stream of nitrogen. The samples were reconstituted in 100 μ L acetonitrile–water (30:70).

2.5. LC-MS/MS method

Separation of the compounds was performed on a Shimadzu Prominence Ultra-Fast Liquid Chromatograph XR System (Shimadzu Benelux, Jette, Belgium) in combination with an Restek Allure PFP Propyl column (50x2.1 mm, 5 μ m) for the screening method, Kinetex® Biphenyl column (150 mm $\times$ 2.1 mm, 2.6 μ m) (Phenomenex, Utrecht, The Netherlands) for method A and an Acquity UPLC® BEH C18 LC Column (50 mm $\times$ 2.1 mm, 1.7 μ m particle size) (Waters, Milford, Massachusetts, United States) for method B. The column oven and autosampler cooler were set at a temperature of respectively 40 °C and 10 °C for all methods.

The screening method used a gradient elution with an aqueous buffer at pH 2.6 (Solvent A) and acetonitrile: 0-10 min: 10-90%, 10-15: 90%, 15-15.5: 90-10%. The total analytical time was 17.5 min. The flow rate was set at 0.5 mL/min with an injection volume of 30 μ l. The flow rate used a gradient profile from 0-10 min: 0.5-1 mL/min; 10-15 min: 1mL/min; 15.50-17.50 0.5 mL/min.

For all matrices, quantitative confirmations were performed on qualitatively identified substances using two separate methods. Method A for citalopram, clomipramine, their respective metabolites, and method B for methadone, and its metabolites.

Method A used a gradient elution with an aqueous buffer at pH 4 (solvent A) and acetonitrile (solvent B): 0-3 min: 30-45%, 3-3.5 min: 45-50%, 3.5- 4 min: 50%, 4-4.5 min: 50-30%, 4.5-6 min: 30%. The system was kept at starting conditions for 5 min to re-equilibrate. The total analytical run time was 11 min. The flow rate was set at 0.7 mL/min with an injection volume of 10 μ L.

Method B used a gradient elution with an aqueous buffer at pH 4 (solvent A) and acetonitrile (solvent B): 0-4 min: 15-95%, 4-5 min: 95-95%, 5- 6 min: 95-15%. The system was kept at starting conditions for 4 min to re-equilibrate. The injection volume was 5 μ L with a flow rate of 0.8 mL/min.

A triple quadrupole MS (3200 QTRAP, Sciex Halle) was operated in scheduled multiple reaction monitoring (sMRM) mode in combination with a Turbo V ion source with positive electrospray ionization (ESI) (Sciex, Halle, Belgium). Following source parameters were set: curtain gas: nitrogen, 25 psi; nebulizing gas: nitrogen, 55 psi; heater gas: nitrogen, 55 psi; ion source temperature: 550 °C; ion source voltage: +5500 V. MRM transitions, retention times and MS parameters are presented in table 1. These MS parameters were determined by direct infusion. The mass spectrometer was coupled to a Dell Precision™ 390 Workstation equipped with Analyst software version 1.5.1. (Sciex, Halle, Belgium) for data acquisition.

2.6. Method validation

The screening method is validated and commercially available as iMethod™ Test for Cliiquid® Software (Sciex, Halle, Belgium). The two quantitative methods were validated in earlier published research for bone tissue and blood [9,20]. A validation step was performed for bone marrow by assessing the following criteria as prescribed by international guidelines^[23]: selectivity, linearity, matrix effect, recovery, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy and stability. For the validation, blank bone marrow was used. This blank bone marrow was taken from forensic cases that tested negative for all compounds using our screening method.

2.6.1. Bone marrow

For methadone, EDDP, EMDP, clomipramine, desmethylclomipramine, citalopram and desmethylcitalopram, matrix-matched calibration curves were created. For analytes, two different calibration curves were created: one for low concentrations (1, 10, 100, 250, 500, 750 and 1000 ng/g) and one for high concentrations (10, 100, 1000, 2500, 5000, 7500, 10000 ng/g) ($n = 5$ at all concentration levels). Different regression models were evaluated: linear least squares un-weighted and weighted ($1/x$, $1/x^2$) regression models and quadratic least squares un-weighted and weighted ($1/x$, $1/x^2$) regression models. The best calibration models were selected based on the lowest back-calculated values. For all analytes, deuterated standards were available and used as internal standards (IS) with exception of desmethylclomipramine and EMPD. For these analytes, respectively clomipramine-d3 and EDDP-d3 were used as an IS. These IS's were selected based on their similar properties during ionization. Precision and accuracy were evaluated using quality control samples at low (1 ng/g) and high concentration (10000 ng/g). Selectivity was tested by analyzing two zero samples from two different donors. LOQ's were set as the lowest points of the calibration

curve, which fulfilled the criteria of sufficient precision and accuracy using spiked quality control samples. The LOD's were estimated using a linear calibration curve containing negative controls ($n = 2$), LOQ ($n = 5$) and the second lowest calibrator ($n = 5$) as described by Polettini et al. (ref). Matrix effect was evaluated by testing a methanolic standard (A) and post-extraction spiked sample (B) at two concentration levels (low and high) using samples from five different donors as described by Matuszewski et al. ^[24]. Recovery was evaluated by testing a methanolic standard (A) and pre-extraction spiked sample (C) at two concentration levels (low and high) using samples from five different donors as described by Matuszewski et al. ^[24]. Processed sample stability was tested by analyzing two samples at high and low concentration after 72 h of storage in the 10°C cooled autosampler.

2.6.2. Bone

Selectivity was tested by analyzing two zero samples from two different donors. For all analytes (100, 200, 300, 600, 750, 1000 ng/g), matrix- matched calibration curves were created ($n = 3$ at all concentration levels). Different regression models were evaluated: linear least squares un- weighted and weighted ($1/x$, $1/x^2$) regression models and quadratic least squares unweighted and weighted ($1/x$, $1/x^2$) regression models.

2.6.3. Blood

Selectivity was tested by analyzing two zero samples from two different donors. For methadone (50, 100, 500, 1000, 2000, 3000, 5000 ng/mL), EDDP (1, 5, 10, 25, 50, 75, 100 ng/mL), clomipramine (10, 100, 500, 750, 1000, 1500, 2000 ng/mL), desmethylclomipramine (10, 100, 500, 750, 1000, 1500, 2000 ng/mL), citalopram (50, 100, 200, 300, 500, 750, 1000 ng/mL) and desmethylcitalopram (50, 100, 200, 300, 500, 750, 1000 ng/mL) matrix- matched calibration curves were created ($n = 3$ at all concentration levels). Different regression models were evaluated: linear least squares un- weighted and weighted ($1/x$, $1/x^2$) regression models and quadratic least squares unweighted and weighted ($1/x$, $1/x^2$) regression models.

3. Results

3.1. Method validation

3.1.1. Bone marrow

Blank samples and zero samples showed no interfering peaks for our analytes. Matrix matched calibration curves were constructed. LODs range from 0.1 ng/g to 0.5 ng/g. LOQ has been set as the lowest calibrator which fulfilled the criteria of sufficient precision and

accuracy using spiked quality control samples. Accuracy expressed as bias (%) was in the proposed acceptance limit for all analytes on all concentration levels and ranged from -14.86 to 10.91% [23]. Repeatability and intermediate precision expressed as relative standard deviations (RSD) (%) ranged respectively from 3.46-19.08% and 7.64-19.89%. All were within the proposed acceptance criteria. The matrix effects ranged from 87.97-122.6%. The recovery ranged from 87.0 – 118.6%. Processed samples were stable in the autosampler, with <10% deviation from starting concentration observed in calculated concentrations up to 72 h post-extraction. Results are summarized in Table 2.

3.1.2. Bone

The method showed no interfering peaks. Calibration curves showed to be linear unweighted for citalopram, desmethylocitalopram, clomipramine and desmethyloclopramine. For methadone, EDDP and EMDP, the best fit showed to be linear with weighing factors of $1/x^2$, respectively. All curves showed good correlation factors ($R > 0.99$).

3.1.3. Blood

The method showed no interfering peaks. Calibration curves showed to be linear unweighted for citalopram, desmethylocitalopram, clomipramine, desmethyloclopramine and EDDP. For methadone, the best fit showed to be linear with weighing factors of $1/x^2$. All curves showed good correlation factors ($R > 0.99$).

3.2. Routine standard toxicological analysis

Using the iMethod™ Test for Cliquid® Software screening method 12 cases were screened for 415 compounds of forensic interest in positive ion mode. A total of 37 unique compounds were identified in all tissues combined. In blood, 37 unique compounds were detected using the protein precipitation and 33 unique compounds using the SPE clean-up. In bone, 29 unique compounds were detected using a methanolic extraction. In bone marrow, 27 unique compounds were detected using SPE.

When comparing the different findings, in 78.4% of blood positive specimens, the corresponding bone was also positive. In 80.5% of blood positive specimens, the corresponding bone marrow was also positive. Four times, a substance (methadone, noscapine, nordiazepam, norfentanyl) was detected in bone and in bone marrow, which was not detected in blood. One time, a substance (nicotine) was only found in bone marrow when

the other matrices were negative. Also, in bone tissue there was one finding (bromazepam) that was not seen in blood nor bone marrow. Supl. Table 1 shows findings for each case. All these findings were confirmed using in-house protocols or quantification methods further specified in this paper.

3.3. Quantification

3.3.1. Clomipramine

For clomipramine, only one case was analyzed. A 32-year-old man had a collapse. He had a known history of taking Anafranil®, Dafalgan® forte, Duovent®, ibuprofen, Leponex®, lorazepam, Lyrica®, Movicol®, Pantomed®, propranolol, Temesta® en Xeplion®.

Clomipramine was identified in bone and bone marrow at concentrations of 256.7 and 5559.1 ng/g, respectively. Blood concentration was 142.0 ng/mL. Its metabolite, desmethylclomipramine, was also detected in concentrations of 17118.1, 900.6 ng/g and 235.2 ng/mL in respectively bone marrow, bone tissue and blood. Results are summarized in table 3.

3.3.2. Citalopram

For citalopram, five cases were analyzed.

Case 2 involves a 27-year-old man with a known history of drug abuse and alcohol addiction that was found dead. In the room where the man was found, fentanyl band-aids, two empty capsules of 'Hawaiian baby woodrose' and sage extract were found.

Citalopram was detected in bone and bone marrow with concentrations of respectively 77.4 and 1561.8 ng/g, respectively. Blood citalopram concentration was 85.2 ng/mL. The metabolite desmethylcitalopram was found in concentrations of 298.6 ng/g in bone marrow, 15.2 ng/g in bone tissue and 104.8 ng/mL in blood.

Case 3 consists of a 52-year-old female nurse found next to her bed wearing a tourniquet. In her house, the following medication was found: insulin, Circadin®, Sipralaxa® en Trazodon®. Post mortem interval was estimated around three days.

Citalopram was detected in bone and bone marrow with concentrations of respectively 45.5 and 1039.6 ng/g, respectively. Blood citalopram concentration was 112.0 ng/mL. The

metabolite desmethylcitalopram was found in concentrations of 161.2 ng/g in bone marrow, 12.4 ng/g in bone tissue and 139.0 ng/mL in blood.

Case 4 concerns a 92-year-old female found dead at the bottom of a staircase.

Concentrations of citalopram and desmethylcitalopram in blood were respectively 2.9 ng/mL and 2.3 ng/mL. Bone concentrations were 1.5 ng/g for citalopram and 2.3 for desmethylcitalopram. Bone marrow concentrations were 14.8 ng/g for citalopram and 25.3 ng/g for desmethylcitalopram.

Case 5 comprises of a 62-year-old male with an alcohol addiction who was found dead on his drive way with a humerus fracture and cardiovascular suffering.

Concentrations of citalopram and desmethylcitalopram in blood were respectively 17.0 ng/mL and 27.4 ng/mL. Bone concentrations were 3.8 ng/g for citalopram and 10.4 for desmethylcitalopram. Bone marrow concentrations were 25.2 ng/g for citalopram and 38.0 ng/g for desmethylcitalopram.

Case 6 deals with a 57 years old female chronic alcohol abuser. Autopsy showed signs of violence and a stomach bleeding.

Concentrations of citalopram and desmethylcitalopram in blood were respectively 19.0 ng/mL and 9.9 ng/mL. Bone concentrations were 2.7 ng/g for citalopram and 1.4 for desmethylcitalopram. Bone marrow concentrations were 37.6 ng/g for citalopram and 16.2 ng/g for desmethylcitalopram.

Results are summarized in table 4. Citalopram concentrations in blood were plotted against the concentrations in bone tissue, the result is shown in be seen in figure 2 and 3..

3.3.3. Methadone

For methadone, six cases were analysed.

In case 7, a 25-year-old male was found dead in bed. He was a daily methadone user. In the house, Tradonal®, Lorazepam®, Seroquel® Lambipol® and cannabis paraphilia were found.

Methadone was detected in bone and bone marrow with concentrations of respectively 13.9 and 39.7 ng/g, respectively. Blood methadone concentration was 2.5 ng/mL. The metabolite

EDDP was not detected in the bone marrow. In bone tissue 1.3 ng/g EDDP was found and 0.6 ng/mL EDDP in blood. The metabolite EMDP was solely detected in bone at a concentration of 0.2 ng/g.

In case 8, a 36-year-old man known for drug crimes, was found dead after smoking cocaine. At the scene of death, Tranxene®, bromazepam and a spoon with white powder were found.

Methadone was detected in bone and bone marrow with concentrations of respectively 0.5 and 7.9 ng/g, respectively. Blood methadone concentration was estimated using muscle tissue around 2.2 ng/mL. The metabolite EDDP was not detected in bone marrow nor in bone tissue. EDDP concentration was estimated around 0.3 ng/mL in blood using muscle tissue. The metabolite EMDP was not detected in this case.

In case 9, an ex-drug user with known alcohol abuse, was found dead on the couch with rib fractures caused by a fight ten days earlier. He presumed to have taken “painkillers” for his fractures.

Methadone was detected in bone and bone marrow with concentrations of respectively 48.0 and 2042.7 ng/g, respectively. Blood methadone concentration was 525.5 ng/mL. The metabolite EDDP was detected in bone marrow at a concentration level of 1208.8 ng/g. EDDP was found at a concentration level of 5.9 ng/g in bone tissue and 71.6 ng/mL in blood. The metabolite EMDP was found in concentrations of 790.5 ng/g in bone marrow, 0.3 ng/g in bone tissue. In blood, EMDP was not detected.

Case 10 concerns a 50-year-old man with known alcohol abuse, which was found in an advanced stage of decomposition in bed. On a nearby table, aluminum foil and pipes were found. In the residence, Xanax®, Lormetazepam, Dominal®, Amlor®, Pantoprazole® and Paracetamol were found.

Methadone was detected in bone and bone marrow with concentrations of respectively 21.4 and 1058.9 ng/g, respectively. In the blood, methadone was present at a concentration of 331.0 ng/mL. The metabolite EDDP was found in concentrations of 1270.7 ng/g in bone marrow, 2.7 ng/g in bone tissue and 68.2 ng/mL in blood. The metabolite EMDP was only found in bone tissue and bone marrow in concentrations of 0.5 and 493.9 ng/g.

Case 11 was a 37-year old male, known as a drug user, who was found dead. He had a known history of using heroin, XTC and speed. At the scene, a bag of white powder was found together with burned spoons and aluminum foil.

Methadone was detected in bone and bone marrow with concentrations of respectively 303.5 and 4218.0 ng/g, respectively. Blood methadone concentration was 3727.4.2 ng/mL. The metabolite EDDP was found in concentrations of 311.8 ng/g in bone marrow, 4.6 ng/g in bone tissue and 32.9 ng/mL in blood. The metabolite EMDP was detected in bone marrow at a concentration of 1573.4 ng/g and in blood at a concentration of 0.1 ng/mL.

In Case 12, a 44-year-old female nurse was found dead in a setting suggesting suicide. Methadone was detected in bone and bone marrow with concentrations of respectively 142.0 and 3775.7 ng/g, respectively. Blood methadone concentration was estimated using muscle tissue around 1742.6 ng/mL. The metabolite EDDP was found in concentrations of 3027.6 ng/g in bone marrow, 36.3 ng/g in bone tissue. EDDP was estimated around 718.5 ng/mL in blood using muscle tissue. The metabolite EMDP was found in concentrations of 1573.5 ng/g in bone marrow. EMDP was not detected in bone and blood tissue.

Results are summarized in table 5. Methadon concentrations in blood were plotted against the concentrations in bone tissue, the result is shown in be seen in figure 4 and 5.

4. Discussion

Collection procedures of skeletal tissue for analysis of drugs have not been standardized. In most published studies, the samples are obtained from random locations. In this study, samples were taken in a standardized fashion. This poses a better starting point to investigate possible correlation. For development of a reference database, a standard procedure is advised since anatomical location has an influence on found drug concentrations.

Routine standard toxicological analysis

Although bone marrow showed higher intensities, most substances were detected using blood. In some cases, metabolites were absent in blood but detected in bone marrow. This can be explained by the higher concentrations in bone marrow. Since a phosphate buffer solution at pH 6 was used for the sample clean-up of blood and bone marrow, not all compounds were extracted. This extraction method was chosen to obtain a good comparison between these two matrices. A simple protein precipitation was not possible for bone marrow due to the high fat content of marrow. This has an influence on the number of compounds detected. Compounds with pKa values under 6 will not be detected using this method. However 80.5% of the time, bone marrow showed to be a valid alternative for blood screening analysis.

When comparing the results of the protein precipitation of blood with the results of the methanolic extraction of bone, less compounds were detected in bone tissue than in blood. This lower performance of bone tissue to detect drugs might be explained by our extraction method. Bones were extracted for 72h at room temperature. This can cause a degradation of certain substances that are not stable at room temperature. However, the detected compounds in blood are stable at room temperature for a short period (72h) ^[25–27]. Another explanation could be that these drugs are simply not incorporated in bone tissue. Aside to that, one can also start to wonder if the long time made assumption that bone tissues show a time frame from the past, is correct ^[28]. Only one substance (bromazepam) was solely detected in bone tissue while it was not present in blood or bone marrow. However, a highly sensitive in house developed method for benzodiazepines showed this substance also to be present in blood ^[29]. As a result, this means that all drugs that were detected in bone tissue, were also detected in blood for our 11 cases. These findings show us that when detecting these substances in bone tissue, a recent intake of this substance might have occurred. In contrast to what was assumed until today ^[28]. Although it is important to bear in mind that the cases were selected based on a positive test for methadone, citalopram and clomipramine. These drugs are meant to be taken chronically to maintain a steady state ^[30,31]. So, detected compounds in bone tissue may represent chronic substance usage. Which in turn would confirm the hypothesis of bone tissue as a drugs depot for chronic substance (ab)use ^[28]. Unfortunately, no medical history was available.

Quantification

Only one case of clomipramine was analyzed. The bone marrow concentration was significantly higher than the others were. This is caused by the high lipophilicity of clomipramine and the fatty texture of bone marrow. Bone marrow was 21.7 times as high as bone concentration and 39.1 as high as blood concentration. The concentration of the metabolite desmethylclomipramine was found to be 19 times higher in bone marrow than in bone tissue and 73 times higher than in blood. The high lipophilicity of bone marrow causes hydrophobic substances to be more easily distributed from the plasma into the bone marrow compared to into bone tissue. These higher concentration in bone marrow make bone marrow more suitable for detecting lipophilic compounds. This shows the high potential of bone marrow when blood is unavailable or concentrations are below the LOD to detect drugs. The bone marrow concentration of desmethylclomipramine was out of our linear range. Therefore, the sample was diluted ½ using blank bone marrow and the concentration was back

calculated. When the drug- to- metabolite relationship is assessed, a ratio of 0.3, 0.3 and 0.6 is seen for respectively bone marrow, bone and blood. This drug-to-metabolite in bone tissue is similar as those seen in rat experiments. The concentration of the more polar metabolite is higher compared to its mother molecule ^[9]. The blood concentrations of clomipramine and desmethylclomipramine are within the therapeutic range ^[32]. Since clomipramine is a drug that should be taken chronically, these values could represent steady state values. However, no data about the last dosing is known.

For citalopram, five cases were analyzed. The bone marrow concentration showed to be the highest followed by respectively the blood concentration and the bone concentration. The high concentration in bone marrow is again due to the high lipophilicity of citalopram. Blood concentrations were within the therapeutic range for case 2, 3, 5, 6^[32]. Case 4 shows a rather low blood concentration, outside of the therapeutic range^[32]. No information is available about the time of the last dosing. For cases 2, 3 and 6, the drug-to-metabolite ratio in bone tissue is similar as those seen in rat experiments. The concentration of the more polar metabolite is lower compared to its mother molecule ^[9]. These could represent steady state values. However, for case 4 and 5, the metabolite is more present than the mother molecule. This could possibly give us some information about the difference between chronic and acute dosing. Unfortunately, no information about the time since last dosing or the dosing pattern was available. When looking at the literature, previous studies showed that it is possible to distinguish acute exposure from chronic exposure for some drugs based on the drug-to-metabolite ratio found in bone tissue. However, this is only shown in animal experiments ^[33]. Direct extrapolation to humans is not possible and our sample size is not big enough to draw conclusions on this.

The correlation between the different matrices has also been investigated. When looking at the ratios of bone concentration over bone marrow concentration, they seem to be in the same range for case 2 & 3. This can be explained by stating that both cases had similar blood concentrations. Therefore, a correlation may be present. When citalopram concentrations in blood were plotted against the concentrations in bone tissue, a trend could be seen. This is shown in figure 2. This trend can be explained by the high vascularization of bone tissue. For the metabolite, the ratios were very variable and no trend could be seen. When plotting the concentrations of citalopram in blood against the concentrations of citalopram in bone marrow, also a trend could be seen. This relation can be seen in figure 3. Several studies

already proposed correlations between blood and bone marrow depending on the substance [17]. In addition, many discrepancies have been found regarding correlation between bone marrow and blood [18,19]. To confirm the possible trend between citalopram levels in blood and in bone marrow, a bigger sample size will be needed. In this way, it will be possible to build a model for the interpretation of toxicological results found in bone marrow.

For methadone, six cases were examined. For case 7, a rib was sampled instead of the clavicle. A ring of 1 cm width was serrated from the third rib, approximately 1 cm from the sternum. For case 8 & 12, no peripheral blood was present due to the heavily decomposed state of the body. Instead, muscle concentrations were used for comparison. Based on a meta-analysis, muscle tissue concentrations are known to be in close concordance with blood concentration [34]. When looking at the median ratio of the concentration of methadone in muscle to that in femoral blood a ratio of 0.74 was observed in the analysis. Therefore muscle tissue was chosen as valid alternative for the absence of blood. This muscle tissue was extracted with the same protocol as bone marrow. The method was not validated for muscle tissue but a deuterated analog of the compound was used as internal standard for quantification. For all cases, the highest concentrations of methadone were found in bone marrow. This is due to the fact that methadone is known to be very lipophilic. EDDP proved not to be detectable in the bone marrow of case 7 & 8 but was detectable in the bone tissue and blood tissue of case 7. In case 8, EDDP was only present in muscle tissue. All found blood concentrations were within the therapeutic range with exception of case 7 & 8 [32]. Cases 7 & 8 were below the therapeutic level, which indicates a dosing more than 24h ago, since methadone has a half-life of 24-48h [30]. When comparing bone and blood concentrations, bone concentrations were lower with exception of case 7. In case 7, the blood concentration was lower than the bone concentration. These findings indicate that a delayed or slow drug absorption may be present. The same mechanism is seen in bone marrow. In bone marrow, drugs are accumulated slowly because of the high lipophilicity [15]. This could have significant implications when interpreting drug screening results for these alternative matrices. Since for case 7 a rib was sampled, another possible explanation can be found in the different sampling site [4]. The drugs to metabolite ratio was also investigated but no correlation could be found. However, when bone methadone concentration was plotted against blood methadone concentration, a linear trend could be seen. This is shown in figure 4. These findings together with our screening results and the results for citalopram concentrations support our theory that drugs found in bone tissue probably show substances that were present in blood at the time of

dead. It contradicts the current assumption that bone tissue gives a window in the history of drugs usage ^[28]. Nevertheless, bone tissue is well protected from outside contamination and could be of great importance when traditional matrices are unavailable ^[35].

The blood methadone concentrations were also plotted against bone marrow methadone concentrations. An exponential trend could be seen between methadone bone marrow concentration and methadone blood concentration. This is shown in figure 5. For diazepam, it is shown in rats that clearance is slower in bone marrow than in blood, giving higher concentrations ^[36]. This accumulation can explain our exponential curve. The same exponential trend is seen when plotting bone methadone concentration against bone marrow concentration. One sample is outside the trend: case 7. This can be explained by the fact that a different bone type was sampled. This indicates the importance of a uniform sampling when looking at bone tissue. For the metabolites, no trends could be seen.

In this research project, the number of cases is limited. This makes it hard to draw decisive conclusions regarding correlation. However, in our results a trend can be seen for citalopram and methadone. Although case 7 and 8 have almost the same blood citalopram level, small variations were present between their respective bone and bone marrow concentrations. The small variations that were seen can be easily explained by biological reasons. Between different individuals, a difference in bone marrow fat content may be observed. Because the fat content of bone marrow is depended of age and/or sex of the deceased^[7]. This in turn can cause a change in drugs concentration since some drugs are more prone to be incorporated in a fattier tissue than others. Our data set consist of a mix of males and females in adulthood. This may cause some variations in the results. Another explanation for variable results can be found in post-mortem changes [16]. Methadone concentrations have been shown to increase in a number of tissue after death indicating the occurrence of post mortem redistribution ^[37]. If post mortem intervals were available, they are reported in the respective table. In this study, the skeletal tissue was relatively fresh. It should be taking into account that bone tissue from remains with advanced decomposition could show a decrease of found drug concentrations. So caution is advised when interpreting skeletal tissue drug concentrations found in heavily decomposed remains.

In literature, another variable that can be found, is the sampling location ^[16]. This was mostly avoided with a uniform sampling method. However, in case 7 a rib was sampled. The results

from case 7 fell slightly out of the general trend seen in figure 4 and 5. This shows the importance of the sampling location. Bearing this in mind, we would like to propose a uniform sampling method so that a reference database can be started. The clavicle bone is easily accessible during autopsy which makes it highly feasible for analysis. However, considerable more work is needed before our method can be adapted as the standard approach.

More research is required before all of the scientific questions associated with skeletal tissue will be answered. There is still a lack of consensus among the active investigators on how to interpret the analysis of drugs in skeletal tissue. A case of Raikos et al. reports found skeletal remains in a forest ^[2]. Back then, no conclusions could be drawn regarding possible intoxication due to an absence of reference material. This case clearly shows the need of having a collection of data relating to drug levels in skeletal tissue. At the moment, reference data is still limited however this project gives an indication of concentration ranges found in post-mortem legal cases and poses a good starting point.

Conclusion

Multiple drugs were successfully identified in all sampled matrices. Less substances were detected in bone (marrow) than in blood but it poses a valid alternative when blood is not available. Especially bone marrow showed big potential with a concordance of 80.5% with blood. For clomipramine, no conclusions can be drawn. To the best of our knowledge, this is the first-time bone (marrow) clomipramine concentrations are been quantified for humans. For the citalopram cases, bone marrow showed the highest citalopram concentrations followed by blood and bone. When plotting blood concentration of citalopram against bone concentrations and bone marrow concentrations, a linear trend could be seen. Methadone was present in all specimens sampled. The metabolites EMDP and EDPP were not detected in some of the samples. The concentrations of methadone in bone marrow were higher than the concentrations in bone tissue. With exception of one case, bone methadone concentrations were higher than the blood concentrations. When plotting the bone marrow methadone concentration against the blood concentration an exponential relation could be seen. When plotting the bone methadone concentration against the blood methadone concentration, a linear relation could be seen. Thus, under the experimental conditions of this study, we have an indication that bone (marrow) concentrations may be used to estimate blood concentrations

for methadone and citalopram. In conclusion, we consider that both bone and bone marrow are of great potential interest and may be useful in determining possible involvement of drugs in forensic toxicology cases.

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Conflict of interest

The authors state that there is no conflict of interest.

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Figure 1. Example of a serrated clavicle bone cleaned of soft tissue.

Figure 2. Relation between bone citalopram concentrations and blood citalopram concentrations based on five post mortem cases

Figure 3. Relation between bone marrow citalopram concentrations and blood citalopram concentrations based on five post mortem cases

Figure 4. Relation between bone methadone concentrations and blood methadone concentrations based on six post mortem cases

Figure 5. *Relation between bone marrow methadone concentrations and blood methadone concentrations based on six post mortem cases*

Table 1. Analyte and deuterated internal standard MS-settings and MRM transitions.

	RT	DP	EP	CEP	Q1 mass	Q3 mass	C E	Q3 mass	CE
	(Min)	(V)	(V)	(V)	(Da)	MRM 1 (Da)	(V)	MR M 2 (Da)	(V)
CLOMIPRAMINE	3.22	36	5.5	10	315. 1	86. 1	27	58.1	53
CLOMIPRAMINE.D3	3.22	41	5.5	16	318. 1	89. 1	27	61.1	55
DESMETHYLCLOMIPRAMINE	3.10	31	5.5	10	301. 2	72.1	25	44.1	63
CITALOPRAM	1.82	46	5	20	325. 0	262.1	27	109.0	33
CITALOPRAM.D6	1.82	46	6.5	14	331. 0	262.1	27	109.0	33
DESMETHYLCITALOPRAM	1.75	46	6	19	331. 0	262.1	27	109.0	33
DESMETHYLCITALOPRAM.D3	1.75	46	6	26	311. 1 314. 1	262.1	27	109.0	33
METHADONE	2.63	40	10	18. 9	310. 2	265.2	20	105.1	35
METHADONE.D3	2.63	40	10	18. 9	313. 2	268.2	20	105.1	35
EDDP	2.44	40	10	17. 9	278. 2	234.2	35	186.0	30
EDDP.D3	2.44	40	10	18. 0	281. 2	234.2	35	/	/
EMDP	3.02	40	10	17. 5	264. 3	234.2	30	/	/

*Underlined transitions were used for quantification. DP: declustering potential; EP: entrance potential; CEP: collision cell entry potential.

Table 2. Validation parameters of the analytical method for bone marrow with coefficients of determination (R^2), limits of quantification (LOQ), limits of detection (LOD), , Matrix effects (ME), Recovery (%), accuracy estimations as bias and precision presented as relative standard deviation values of repeatability (RSD_{rep}) and intermediate precision ($RSD_{int pr}$)

						Low							High						
	Range (ng/g)	Calibration model	R ²	LOD (ng/g)	LOQ (ng/g)	Bias (%)	RS D _{rep} (%)	RS D _{int} pr (%)	ME (%)	RS D _M E (%)	RE (%)	RS D _{RE} (%)	Bias (%)	RS D _{rep} (%)	RS D _{int} pr (%)	ME (%)	RS D _M E (%)	RE (%)	RS D _{RE} (%)
Clomipramine	1 – 1000 1000-10000	Quadratic Linear 1/X	0.998 0.998	0.1	1	2.28	8.85	15.86	12.26	11.9	87.0	9.6	-14.86	4.48	7.65	10.71	3.14	88.5	5.4
Desmethyldesmipramine	1 – 1000 1000-10000	Quadratic Linear	0.980 0.994	0.5	1	-8.72	19.08	19.89	11.07	18.0	10.17	13.4	10.09	5.19	10.5	10.36	3.63	10.4	8.3
Citalopram	1 – 1000 1000-10000	Linear Linear 1/X ²	0.998 0.996	0.1	1	5.11	8.84	19.63	81.97	13.4	10.42	13.9	-1.42	11.40	14.8	10.37	3.79	89.2	13.7
Desmethylocitalopram	1 – 1000 1000-10000	Linear 1/X ² Linear 1/X	0.995 0.997	0.1	1	10.91	16.19	19.68	79.7	18.5	10.71	14.7	2.44	11.36	14.0	11.09	9.63	10.27	14.6
Methadone	1 – 1000 1000-10000	Linear 1/X Linear	0.998 0.998	0.1	1	-12.21	3.46	9.23	10.22	1.41	98.3	4.3	-1.44	6.17	7.98	98.93	1.34	10.60	3.7
EDDP	1 – 1000 1000-10000	Quadratic Linear	0.993 0.998	0.1	1	-1.89	15.21	19.53	85.54	6.62	92.7	3.9	0.82	5.79	7.64	10.00	0.66	10.60	4.6

	00 0																		
EMDP	1 – 10 00 10 00- 10 00 0	Linea r Linea r	0.9 98 0.9 99	0.3	1	- 1.7 2	9.6 3	14. 86	11 3.2	9.5 9	89. 5	5.4	- 1.3 6	8.2 5	13. 3	11 2.0	1.9 5	11 8.6	5.5

Table 3. Concentrations found in each biological matrix for clomipramine and its metabolite

	Blood (ng/mL)		Bone (ng/g)		Bone marrow (ng/g)				
	Clomipr amine	Desmethylclo mipramine	Clomipr amine	Desmethylclo mipramine	Clomipr amine	Desmethylclo mipramine	Gen der	A ge	P MI
Ca se 1	142.0	235.1	256.6	900.6	5559.1	17118.1	Mal e	32	68 h

Table 4. Concentrations found in each biological matrix for citalopram and its metabolite

	Blood (ng/mL)		Bone (ng/g)		Bone marrow (ng/g)				
	Citalopram	Desmethyleitalopram	Citalopram	Desmethyleitalopram	Citalopram	Desmethyleitalopram	Gender	Age	PMI
Case 2	491.8	77.3	77.4	15.2	1527.7	312.3	Male	27	24-48h
Case 3	348.3	138.0	45.5	12.4	1017.1	138.0	Female	52	72h
Case 4	1.4	2.3	1.5	2.3	12.1	25.7	Female	92	48-72h
Case 5	17.0	27.4	3.8	10.4	25.2	38.0	Male	62	24-36h
Case 6	19.0	9.9	2.7	1.4	37.6	16.2	Female	57	24-48h

Table 5. Concentrations found in each biological matrix for methadone and its metabolites

	Blood (ng/mL)			Bone (ng/g)			Bone marrow (ng/g)					
	Methadone	EDDP	EMDP	Methadone	EDDP	EMDP	Methadone	EDDP	EMDP	Gender	Age	PMI
Case 7	2.5	0.6	n.d.	13.9	1.3	0.2	39.7	n.d.	n.d.	Male	25	48h
Case 8	2.2*	0.3*	n.d.*	0.5	n.d.	n.d.	7.9	n.d.	n.d.	Male	50	72h
Case 9	525.5	71.6	n.d.	48.0	5.9	0.3	2042.7	1208.8	790.5	Male	25	24h
Case 10	331.0	68.2	n.d.	21.4	2.7	0.5	1058.9	1270.7	493.9	Male	36	/
Case 11	3727.4	32.9	0.1	303.5	4.6	0.4	4218.0	311.8	9.9	Male	37	/
Case 12	1742.6*	718.5*	n.d.*	142.0	36.3	n.d.	3775.7	3027.6	1573.4	Female	44	48-72h

n.d. = Not detected

* muscle concentration