# Distribution of clomipramine, citalopram, midazolam, and metabolites in skeletal tissue after chronic dosing in rats

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# Distribution of clomipramine, citalopram, midazolam, and metabolites in skeletal tissue after chronic dosing in rats

Michiel Vandenbosch | Tomas Somers | Eva Cuypers

Toxicology and Pharmacology, KU Leuven, Leuven, Belgium

**Correspondence**
Eva Cuypers, Department of Pharmaceutical and Pharmacological Sciences, Toxicology and Pharmacology, University of Leuven (KU Leuven), Campus Gasthuisberg, O&N II, P.O. Box 922, Herestraat 49, 3000 Leuven, Belgium.
Email: eva.cuypers@kuleuven.be

## Abstract

In recent years, the use of skeletal tissue as an alternative matrix in forensic toxicology has received new interest. In cases where extreme decomposition has taken place, analysis of skeletal tissue is often the only option left. In this article, a fully validated method is presented and the distribution of clomipramine, citalopram, midazolam, and metabolites after chronically administration is examined within skeletal tissue. Rats were chronically dosed with respectively clomipramine, citalopram, or midazolam. Extracts were quantitatively analyzed using liquid chromatography –electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS). Clomipramine, citalopram, and metabolites, respectively desmethylclomipramine and desmethylcitalopram are shown to be detectable in all bone types sampled. Midazolam and its metabolite α-OH-midazolam could not be detected. The absence of midazolam in extracts gives an indication that drugs with pKa values under physiological pH are badly or not incorporated in bone tissue. Bone and post-mortem blood concentrations were compared. A range of different bone types was compared and showed that the concentration is strongly dependent on the bone type. In concordance with previous publications, the humerus shows the highest drug levels. Skeletal tissue concentrations found ranged from 1.1 to 587.8 ng/g. Comparison of the same bone type between the different rats showed high variances. However, the drugs –metabolite ratio proved to have lower variances (<20%). Moreover, the drugs –metabolite ratio in the sampled bones is in close concordance to the ratios seen in blood within a rat. From this, we can assume that the drugs–metabolite ratio in skeletal tissue may prove to be more useful than absolute found concentration.

**KEYWORDS**

chronic dosage, skeletal tissue

## 1 | INTRODUCTION

In recent years, the usage of skeletal tissue as an alternative matrix in forensic toxicology has received new interest. Skeletal tissue is seen as an advantageous matrix for drug testing for a number of reasons. In cases where extreme decomposition has taken place, bone analysis is often the only option left.[1] Additionally, it is assumed that analysis of skeletal tissue could provide a unique retrospective window of drugs detection.[2] This characteristic might provide additional information for the interpretation of drug findings in other matrices like blood and urine. For example, in a post-mortem case where high amounts of benzodiazepines are found, it is important to know whether the patient had developed tolerance toward the drug due to chronic abuse. Skeletal tissue may provide an answer to this question.

The human skeleton makes up a total of 213 bones. Human bones can be classified into long, flat, short, and irregular bones.[3,4] Previous

  

research has already shown that long bones yield higher drug concentrations and thus are more favorable when collecting samples.[5,6] An explanation is found in the higher vascularization of these bones.[4] A long bone can be divided into two major parts. The middle part is made up of a hollow shaft, the diaphysis. The diaphysis consists mostly of cortical bone. Cortical bone is compact with dense bone tissue giving the strength to the skeleton. Inside the hollow shaft of the diaphysis, bone marrow can be found. Bone marrow is the primary site of blood cell production and is known to be a depot for certain drugs. On either side of this hollow shaft, the epiphysis is found. The epiphysis mostly consists of a different kind of bone tissue, trabecular bone. Trabecular or spongy bone is more porous, and the structure can be compared to a honeycomb. Inside these pores, very often bone marrow and vascularization can be found. Due to this structure, cancellous bone has a bigger contact surface area with vascularization and bone marrow compared to trabecular bone, which makes it more suitable for drug-to-bone interaction. This bigger contact surface explains the higher drug concentration found in the epiphysis compared to the diaphysis.[7] The exact mechanism of how drugs are incorporated in skeletal tissue is still under discussion. A recent model gives a possible explanation for the incorporation of drugs into bone tissue.[8] Xenobiotics are possibly incorporated into bones through the channels of Haver and Volkmann, which house the vascularization of bone tissue. In these channels, a local equilibrium will form due to ion exchange between the hydrated layer of the bone tissue and the vascular fluids. In this hydrated layer, xenobiotics are mineralized in the crystal bone structure through substitution or chelation. Since there are differences in the degree of vascularization within and between bones, the model partially explains the difference in found drug concentrations within a bone and between bones. Although this proposed model gives a better view on the mechanism behind drugs incorporation, there is still research needed to study the actual distribution of drugs into the skeletal tissue.

Over the last few years, many studies have been published dealing with extraction methodologies and detection of drugs in skeletal tissue which mostly apply mass spectrometry (MS) or tandem MS (MS/MS techniques coupled to either gas chromatography (GC) or liquid chromatography (LC).[9-11] Despite the growing knowledge about this matrix, interpretation of the found concentrations remains very difficult. To have a basis for interpreting analytical results from skeletal tissue, all different factors that influence bone drug concentrations need to be investigated. Biological factors and environmental factors may play a role but as a starting point, factors concerning dosage and frequency of usage should be evaluated.[12,13]

In this article, a fully validated method is presented to quantify the distribution of clomipramine, citalopram, midazolam, and their metabolites in bone tissue of chronically dosed Wistar rats. These drugs are chosen based on their forensic interest and are commonly seen in autopsy cases.[14] Clomipramine is a tricyclic antidepressant (TCA).[15,16] TCAs were the first antidepressants to make it to the market. It is most common used as a treatment for depression and obsessive compulsive disorder. Citalopram is also an antidepressant but it works as a selective serotine reuptake inhibitor (SSRI).[15,17] This group of antidepressants is the most commonly used and is seen as the golden standard nowadays. Midazolam is a short-acting benzodiazepine with strong relaxing effects.[18,19] It is often prescribed as medication for troubled sleepers or severe agitation. It can also be used as a treatment for seizures. These classes of drugs are frequently detected in forensic screenings. They are relatively safe but can be dangerous in combination with other drugs or alcohol.[15] In forensic toxicological cases, these drugs are also frequently linked to suicide.[14,20] In the past, many different extraction procedures have been described for components in bone tissue. In our study, a simple extraction procedure was applied on full bones using methanol.[10,21] Drug levels in different bones from various anatomical body sites of chronic-dosed rats were compared and the accumulation of drugs and their metabolites were assessed in these sites. These drugs levels are also compared to found blood concentrations.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and reagent

Cipramil® 40 mg/mL (citalopram. HCl) was obtained from Lundbeck NV (Brussels, Belgium); Anafranil® 25 mg/2 mL (clomipramine. HCl) was obtained from Defiante Farmacêutica (Funchal, Portugal); and Dormicum® 5 mg/5 mL (midazolam) was obtained from NV Roche SA (Brussels, Belgium).

Analytical reference standards of clomipramine (1 mg/mL), clomipramine.d3 (100 μg/mL), desmethylclomipramine (1 mg/mL), citalopram (1 mg/mL), citalopram.d6 (100 μg/mL), desmethylcitalopram (1 mg/mL), desmethylcitalopram.d3 (100 μg/mL), midazolam (1 mg/mL), midazolam.d4 (100 μg/mL), α-OH-midazolam(1 mg/mL), and α-OH-midazolam.d4 (100 μg/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 high performance liquid chromatography (HPLC) grade. Acetonitrile and methanol were obtained from Biosolve (Valkenswaard, Netherlands). 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). Aqueous buffer was prepared as follows: 10mM of ammonium formate, adjusted to pH 3 with formic acid.

### 2.2 | Animal model

Male Wistar rats ($n$ = 23) weighing 320–380 g and aged between 80 and 87 days were obtained from Charles River Laboratories (Elsene, Belgium). The animals were housed at the Animal Facility Gasthuisberg of the University of Leuven. Upon arrival at the facility, the animals were allowed at least 7 days to acclimatize to the conditions. They were housed in groups of two in Macrolon cages on a 12-hour light–dark cycle at an ambient temperature of 20–22°C. The animals were supplied with food and water ad libitum. The

animals were divided into four groups. Three groups respectively received 13 mg/kg of citalopram ($n$ = 6), 12.5 mg/kg of clomipramine ($n$ = 5), and 0.33 mg/kg of midazolam (n = 6). One group functioned as control and received saline (n = 6). Once daily, they were injected subcutaneously (SC) for a period of 139 days (except for midazolam, which was injected for only 109 days. Approximately 24 hours after the last injection, animals were euthanized using $CO_2$.

The daily doses were derived using allometric calculations and the maximal recommended tolerated dose (MRTD) for humans as recommended by the Food and Drug Administration (FDA).[22] All drug preparations were diluted using saline to create an injection volume of 0.6–0.8 mL. Twenty-four hours after the last injection, the animals were euthanized using $CO_2$. This experiment was approved by the Ethical Committee Animal Experimentation of the University of Leuven (P 113/2011).

## 2.3 | Specimen preparation

### 2.3.1 | Blood

After the euthanization of dosed rats and the control group, blood was instantly collected in tubes containing sodium fluoride. One hundred µL of this blood was spiked with 10 µL of clomipramine.d3 (1 µg/mL), 10 µL of citalopram.d6 (1 µg/mL), 10 µL of desmethylcitalopram.d3 (1 µg/mL), 10 µL of midazolam.d4 (1 µg/mL), and 10 µL of OH-midazolam.d4 (100 ng/mL) followed by a protein precipitation using 200 µL acetonitrile. This mixture was centrifuged for 10 minutes at 2500 rpm. Afterwards, 100 µL supernatant was transferred to a vial and analyzed using liquid chromatography coupled to a triple quad mass spectrometer with an electron spray ionization source (LC-ESI(+)-MS/MS).

Matrix-matched calibration curves were prepared by spiking 1 mL blank human blood at different concentrations. Blank donor whole blood was supplied by the Blood Transfusion Center (Gasthuisberg, Leuven, Belgium). Zero samples were prepared by spiking 100 µL of blank blood with 10 µL of clomipramine.d3 (1 µg/mL), citalopram.d6 (1 µg/mL), desmethylcitalopram (1 µg/mL), midazolam.d4 (1 µg/mL), OH-midazolam.d4 (100 ng/mL). These samples are processed as described in section 2.3.1.

### 2.3.2 | Skeletal tissue

Femora, claviculae, tibiae, ulnae, radii, humeri, and scapulae were removed by dissection. These bones were cleaned by scraping the attaching soft tissue off with a scalpel. Afterwards, claviculae, femora, tibiae, ulnae, radii, humeri, and scapulae were extracted by full submersion in respectively 1, 5, 6, 4, 3, 4, and 4 mL methanol at room temperature for 72 hours. At the start of the extraction, single full bones were spiked with internal standards by addition of 50 ng clomipramine.d3, 50 ng citalopram.d6, 50 ng desmethylcitalopram.d3, 50 ng midazolam and 10 ng α-OH-midazolam.d4 in the extraction solvent. After removal of the bone, the solution was evaporated under $N_2$ and reconstituted in 100 µL mixture of 70:30 $H_20$:ACN. This solution was centrifuged for 10 minutes at 3000 rpm at 0°C. The top 50 µL methanol was taken off and transferred to a vial to avoid possible fatty textures in the final extract.

For the method validation, quality control samples were prepared by spiking a 100 mg cut-of piece of rat bone with a standard stock solution of clomipramine, desmethylclomipramine, citalopram, desmethylcitalopram, midazolam, and α-OH-midazolam at concentrations as shown in Table 2. Blank rat skeletal tissue was obtained by dissection of the control group. Zero samples were prepared by spiking blank rat skeletal tissue with 50 ng clomipramine.d3, 50 ng citalopram.d6, 50 ng desmethylcitalopram.d3, 50 ng midazolam.d4 and 10 ng OH-midazolam.d4. These samples are processed as described in 2.3.2.

## 2.4 | 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 Kinetex® Biphenyl column (150 mm × 2.1 mm, 2.6 µm) (Phenomenex, Utrecht, Netherlands). The method used gradient elution with an aqueous buffer at pH 4 (solvent A) and acetonitrile (solvent B): 0–3 minutes: 30%–45%, 3–3.5 minutes: 45%–50%, 3.5–4 minutes: 50%, 4–4.5 minutes: 50%–30%, 4.5–6 minutes: 30%. The system was kept at starting conditions for 5 minutes to re-equilibrate. The total analytical run time was 11 minutes. The flow rate was set at 0.7 mL/min with an injection volume of 10 µL. The column oven and autosampler cooler were set at a temperature of 40°C and 10°C, respectively.

A triple quadrupole MS (3200 QTRAP, Sciex, Halle, Belgium) 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). The 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.

The extracts were analyzed in sMRM mode for clomipramine, Desmethylclomipramine, citalopram, desmethylcitalopram, midazolam, and OH-midazolam. The deuterated internal standards (ISs) were used to quantify analytes and their metabolites. For all analytes, deuterated standards were available and used as internal standards with the exception of desmethylclomipramine. For this analyte, clomipramine.d3 was used as an IS. This IS was selected based on its similar properties during ionization.

## 2.5 | Method validation

A validation step was performed by assessing the following criteria as prescribed by international guidelines[23]: selectivity, linearity, matrix

**TABLE 1** MRM transitions of precursor ions (Q1), product ion (Q3) with respectively retention time (Rt), declustering potential (DP); entrance potential (EP); collision cell entry potential (CEP)

| | Rt (min) | DP (V) | EP (V) | CEP (V) | Q1 Mass (Da) | Q3 Mass MRM 1 (Da) | CE (V) | Q3 Mass MRM 2 (Da) | CE (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 | 311.1 | 262.1 | 27 | 109.0 | 33 |
| Desmethylcitalopram.d3 | 1.75 | 46 | 6 | 26 | 314.1 | 262.1 | 27 | 109.0 | 33 |
| Midazolam | 1.72 | 66 | 5.5 | 14 | 326.2 | 291.1 | 33 | 249.0 | 44 |
| Midazolam.d4 | 1.72 | 66 | 5.5 | 14 | 330.2 | 295.1 | 33 | 253.0 | 44 |
| OH-midazolam | 1.58 | 51 | 11 | 19 | 342.2 | 324.0 | 27 | 203.0 | 35 |
| OH-Midazolam.d4 | 1.59 | 51 | 11 | 19 | 346.2 | 328.0 | 27 | 203.0 | 35 |

*Underlined transitions were used for quantification.

**TABLE 2** Concentrations of quality control samples for skeletal tissue

| | Low (ng/g) | Medium (ng/g) | High (ng/g) |
|---|---|---|---|
| Clomipramine | 1 | 100 | 200 |
| Citalopram | 1 | 100 | 200 |
| Desmethylcitalopram | 1 | 100 | 200 |
| Midazolam | 1 | 100 | 200 |
| OH-midazolam | 0.5 | 10 | 20 |
| Desmethylclomipramine | 0.5 | 10 | 20 |

effect, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, and stability.

### 2.5.1 | Skeletal tissue

For skeletal tissue, the validation was performed similar as described in Vandenbosch et al.[5] For clomipramine, citalopram, midazolam, and desmethylcitalopram, matrix-matched calibration curves were created (1, 5, 10, 50, 100, 150, 300 ng/g) and for OH-midazolam and desmethylclomipramine (0.5, 1, 5, 10, 15, 20 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. The concentrations of quality control samples used to evaluate precision and accuracy can be found in Table 2.

### 2.5.2 | Blood

A partial validation was performed for blood. Selectivity was tested by analyzing two zero samples from two different donors. For clomipramine, midazolam (0.5, 1, 10, 25, 50, 75, 100 ng/mL), OH-midazolam, desmethylclomipramine (0.5, 1, 2, 4, 8, 12, 16 ng/mL), citalopram (0.75, 2, 3, 6, 12, 24, 36, 48 ng/mL), and desmethylcitalopram (0.75, 2, 3, 6, 12, 24, 36, 48 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/x2) regression models and quadratic least squares unweighted and weighted (1/x, 1/x2) regression models. The best calibration models were selected based on the lowest back-calculated values.

## 3 | RESULTS

### 3.1 | Method validation

### 3.1.1 | Skeletal tissue

Figure 1 shows an example of a chromatogram of an extracted spiked bone at high level concentrations. The validation results are summarized in Table 3. Blank samples and zero samples showed no interfering peaks for our analytes. Matrix matched calibration curves were constructed and yielded quadratic correlations with weighting factors of $1/x^2$ for each analyte with the exception of citalopram, which was unweighted quadratic. LODs range from 0.1 ng/g to 0.3 ng/g. LOQ was 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 −17.9 to 17.8%.[23] Repeatability and intermediate precision expressed as relative standard deviations [RSD (%)] ranged respectively from 2.84 to 18.5% and 2.94 to 17.1%. All were within the proposed acceptance criteria with the exception of the intermediate precision of midazolam at high concentration level. The acceptance limit was exceeded by 2%. However, repeatability and bias proved to be good for this level. The matrix effects ranged from 36.78 to 107.9%. Recovery ranged from

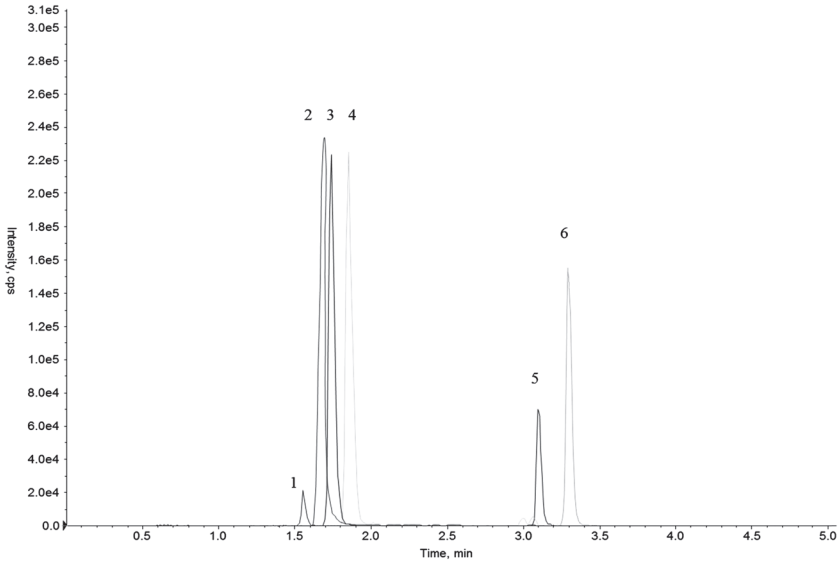


**FIGURE 1** Chromatogram of an extracted spiked bone at high level concentrations with OH-midazolam (1), midazolam (2), desmethylcitalopram (3), citalopram (4), desmethylclomipramine (5), and clomipramine (6), Respectively.

35.05 to 94.73%. Processed samples were stable in the autosampler, with <20% deviation from starting concentration observed in calculated concentrations up to 72 hours post-extraction.

### 3.1.2 | Blood

The method showed no interfering peaks. Calibration curves showed to be linear unweighted for citalopram, midazolam, and OH-Midazolam. For desmethylcitalopram and desmethylclomipramine, the best fit showed to be quadratic with weighing factors of 1/x and $1/x^2$, respectively. All curves showed good correlation factors ($R > 0.99$). LODs were estimated around 0.1 ng/mL for all compounds with the exception of OH-midazolam which was estimated around 0.3 ng/mL. LOQs were set as the lowest calibrator with sufficient precision at 0.5, 0.75, 0.5, 0.75, 0.5, and 0.5 ng/mL for desmethylcitalopram, citalopram, clomipramine, desmethylclomipramine, midazolam, and OH-midazolam, respectively.

## 3.2 | Distribution in skeleton

The distribution of clomipramine, citalopram, and midazolam and their metabolites were studied in the skeletal tissue of chronically dosed rats. All sampled bones tested positive for quantifiable amounts of their respective administered drug and corresponding metabolite with the exception of those dosed with midazolam.

### 3.2.1 | Clomipramine

The skeletal concentrations found in the rats dosed with clomipramine can be found in Figure 2. Concentrations for clomipramine ranged from 25.4 to 296.1 ng/g. In general, concentrations of its metabolite desmethylclomipramine were lower, ranging from 1.1 to 20.5 ng/g. Mean concentrations were calculated for each rat and across all the rats for each bone type separately. The variance was evaluated as RSD (%). Within a rat, a big variance between the sampled bones was seen ranging from 35.7 to 59.9% for clomipramine and from 36.7 to 65.4% for desmethylclomipramine. Comparison of the same bone type between rats showed variances of 23.7%–47.3% for clomipramine and 16.6%–42.3% for desmethylclomipramine. The ratios drug/metabolite were also assessed. Mean ratios were calculated within rats as well as across all the rats for each bone type separately and the variance was evaluated as RSD (%). Within a rat, the mean ratio ranged from 11.1 to 18.0 with variances of 11.5%–17.2%. Between-rat comparisons of the mean ratio of the same bone type ranged from 12.9 to 16.8 with variances ranging between 12.7 and 24.3%. Drug to metabolite ratios for clomipramine can be seen in Figure 3. The found bone concentrations, the drug/metabolite ratios, calculated means, and RSDs can be found in Table S1.

For clomipramine, blood concentrations for the five rats ranged from 39.1 to 70.1 ng/mL and 1.1 to 4.2 ng/mL for desmethylclomipramine. They had a variance of 30.2% and 34.5%, respectively. Ratios of clomipramine to desmethylclomipramine ranged from 10.9 to 26.0 with a variance of 29.5%.

### 3.2.2 | Citalopram

The skeletal concentrations found in the rats dosed with citalopram can be found in Figure 4. For citalopram, concentrations ranged from 5.4 ng/g to 303.6 ng/g. They were lower compared to its metabolite desmethylcitalopram which ranged from 4.1 ng/g to 587.8 ng/g. Two values of desmethylcitalopram proved to be outside our linear range and are considered as semi-quantitative. The variance was evaluated as RSD (%). Within a rat, a big variance between the sampled bones was seen, ranging from 47.3 to 86.8% for citalopram and from 46.9 to 86.1% for desmethylcitalopram. Comparison of the same bone type between rats showed variances of 63.8%–93.9% for citalopram and 66.4%–107% for desmethylcitalopram. The ratios

**TABLE 3** Validation parameters of the analytical method for skeletal tissue with coefficients of determination ($R^2$), limits of quantification (LOQs), limits of detection (LODs), matrix effects (MEs), recovery (RE), accuracy estimations as bias and precision presented as relative standard deviation values of repeatability ($RSD_{rep}$), and intermediate precision ($RSD_{int\ pr}$)

| | | | | | Low | | | | | | | Medium | | | High | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Range (ng/g) | Calibration model | $R^2$ | LOD (ng/g) | LOQ (ng/g) | Bias (%) | $RSD_{rep}$ (%) | $RSD_{int\ pr}$ (%) | ME (%) | $RSD_{ME}$ (%) | RE (%) | $RSD_{RE}$ (%) | Bias (%) | $RSD_{rep}$ (%) | $RSD_{int\ pr}$ (%) | Bias (%) | $RSD_{rep}$ (%) | $RSD_{int\ pr}$ (%) | ME (%) | $RSD_{ME}$ (%) | RE (%) | $RSD_{RE}$ (%) |
| 1–300 | Quadratic $1/X^2$ | 0.992 | 0.1 | 1 | 17.8 | 18.5 | 12.8 | 79.84 | 7.22 | 85.95 | 1.63 | 8.49 | 5.73 | 9.03 | −6.08 | 3.54 | 12.8 | 93.28 | 5.10 | 85.95 | 1.63 |
| 0.5–20 | Quadratic $1/X^2$ | 0.990 | 0.2 | 0.5 | 1.74 | 13.0 | 12.3 | 94.96 | 7.43 | 59.05 | 14.0 | −0.83 | 3.51 | 2.94 | 12.8 | 8.92 | 10.6 | 94.68 | 6.29 | 88.66 | 7.85 |
| 1–300 | Quadratic | 0.999 | 0.2 | 1 | −17.9 | 8.48 | 7.01 | 77.26 | 12.6 | 62.17 | 9.73 | 10.8 | 6.99 | 11.7 | −15.9 | 3.12 | 13.5 | 92.73 | 6.54 | 94.73 | 4.11 |
| 1–300 | Quadratic $1/X^2$ | 0.994 | 0.1 | 1 | −1.86 | 13.8 | 12.3 | 104.5 | 8.20 | 80.61 | 3.99 | 14.1 | 5.38 | 11.2 | −1.33 | 2.84 | 6.22 | 96.95 | 3.94 | 80.61 | 3.99 |
| 1–300 | Quadratic $1/X^2$ | 0.994 | 0.3 | 1 | −14.4 | 9.81 | 8.46 | 99.25 | 12.3 | 69.13 | 8.28 | −1.41 | 6.89 | 13.7 | −3.30 | 5.26 | 17.1[a] | 107.9 | 5.92 | 80.65 | 1.58 |
| 0.5–20 | Quadratic $1/X^2$ | 0.985 | 0.3 | 0.5 | −10.5 | 15.4 | 13.7 | 36.78 | 10.5 | 35.05 | 6.87 | −6.70 | 7.45 | 9.22 | −6.70 | 7.45 | 9.22 | 65.95 | 10.1 | 57.10 | 9.08 |

[a]Marks values that transgress the acceptance threshold.

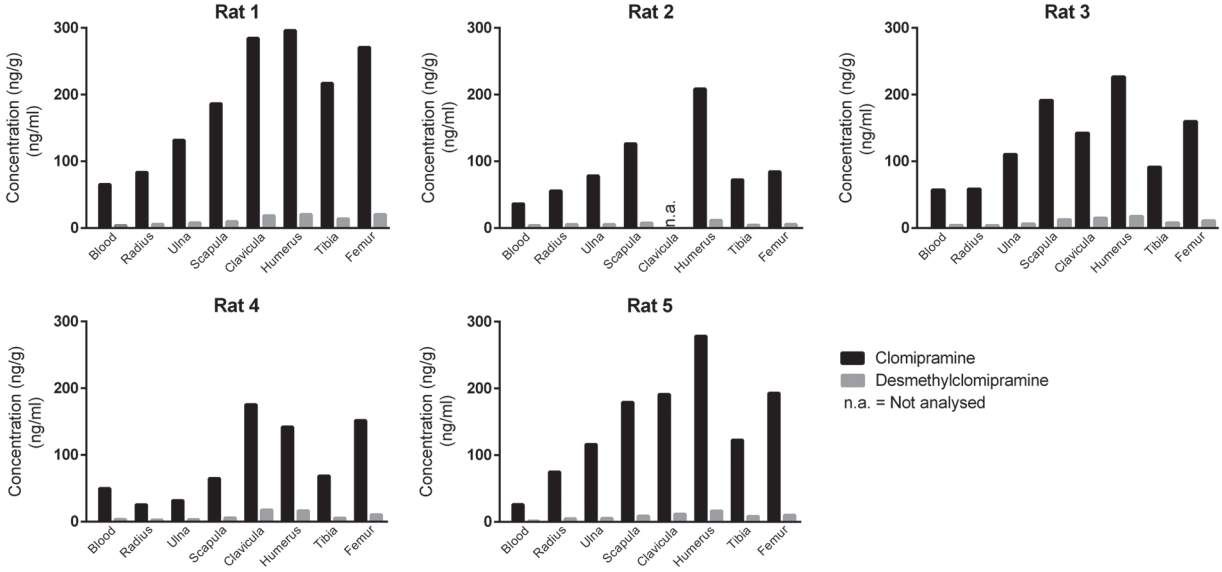


**FIGURE 2** Distribution of clomipramine and its metabolite desmethylclomipramine for each bone type

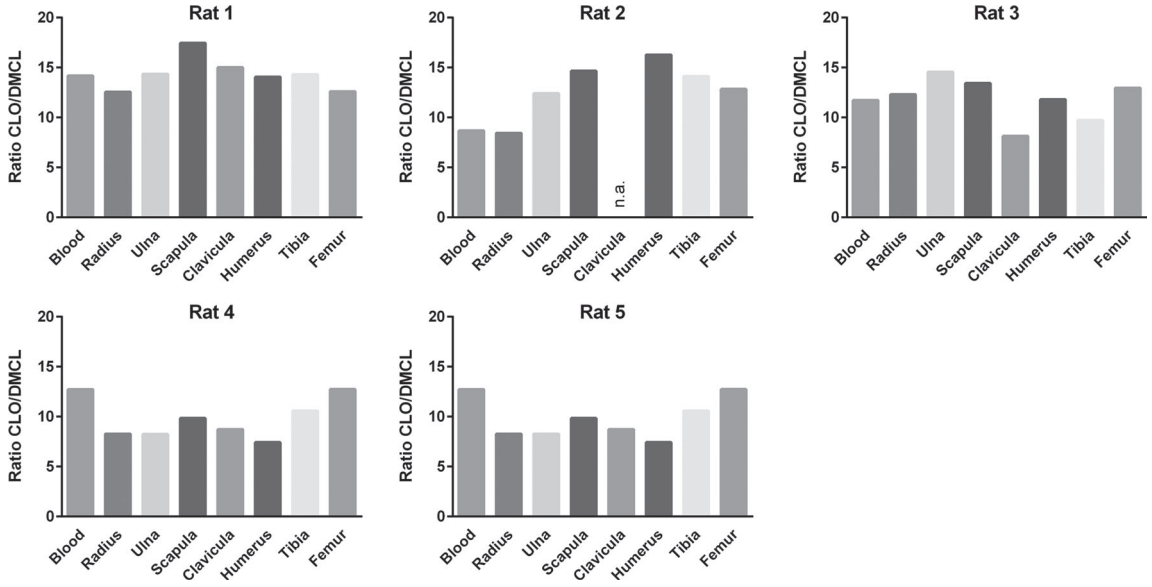


**FIGURE 3** The relative drug–metabolite ratio for clomipramine/desmethylclomipramine for each bone type

drug/metabolite were also assessed. Mean ratios were calculated within rats as well as across all the rats for each bone type separately and the variance was evaluated as RSD (%). Within a rat, the mean ratio ranged from 0.39 to 2.09 with a variance ranging between 11.1 and 16.6%. Between rats, comparison showed the mean ratio of the same bone type to range from 0.75 to 1.02 with variances of 54.8 to 82.2%. Drug-to-metabolite ratios for citalopram can be seen in Figure 5. The found bone concentrations, the drug/metabolite ratios, calculated means and RSDs can be found in Table S2.

For citalopram, blood concentrations for the six rats ranged from 10.3 to 64.0 ng/mL and from 8.6 to 117.6 ng/mL for desmethylcitalopram. A variance of 76% and 75%, respectively, was observed. Ratios of citalopram to desmethylcitalopram ranged from 0.32 to 1.73. They varied by approximately 65.1%.

### 3.2.3 | Midazolam

Midazolam and its metabolites showed to be absent in all bones sampled using this method. This method was also tested after grinding the bone in order to expose bone marrow but no midazolam or the metabolite was detected. Blood samples were also shown to be negative for midazolam and its metabolites, using the developed method across all rats.

## 4 | DISCUSSION

In this project, the distribution pattern of three different drugs with forensic relevance and their metabolites are studied in the skeleton

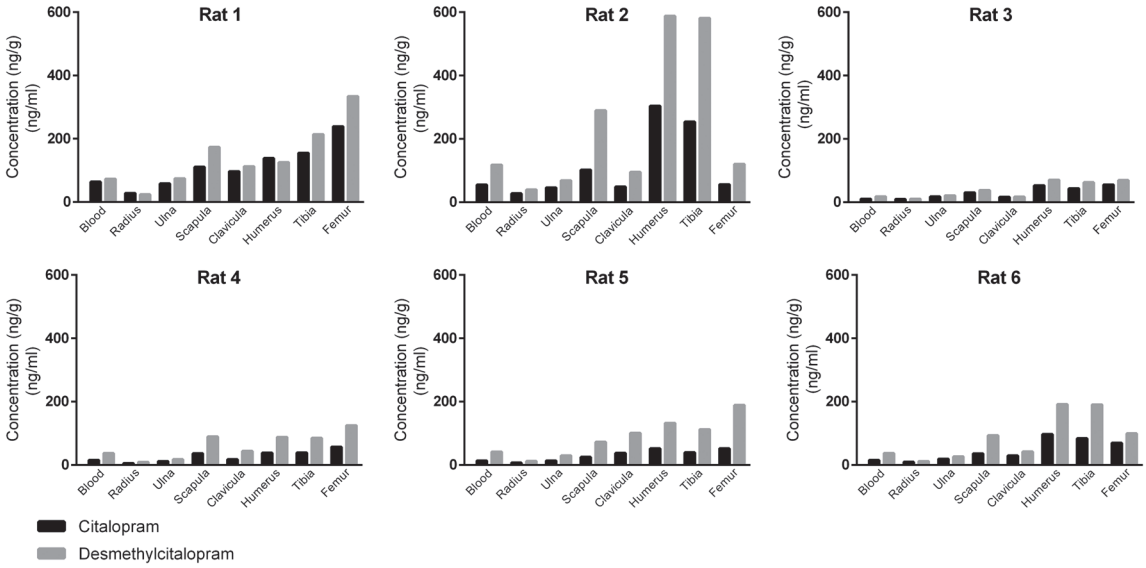


**FIGURE 4** Distribution of citalopram and its metabolite desmethylcitalopram in skeletal tissue for each bone type

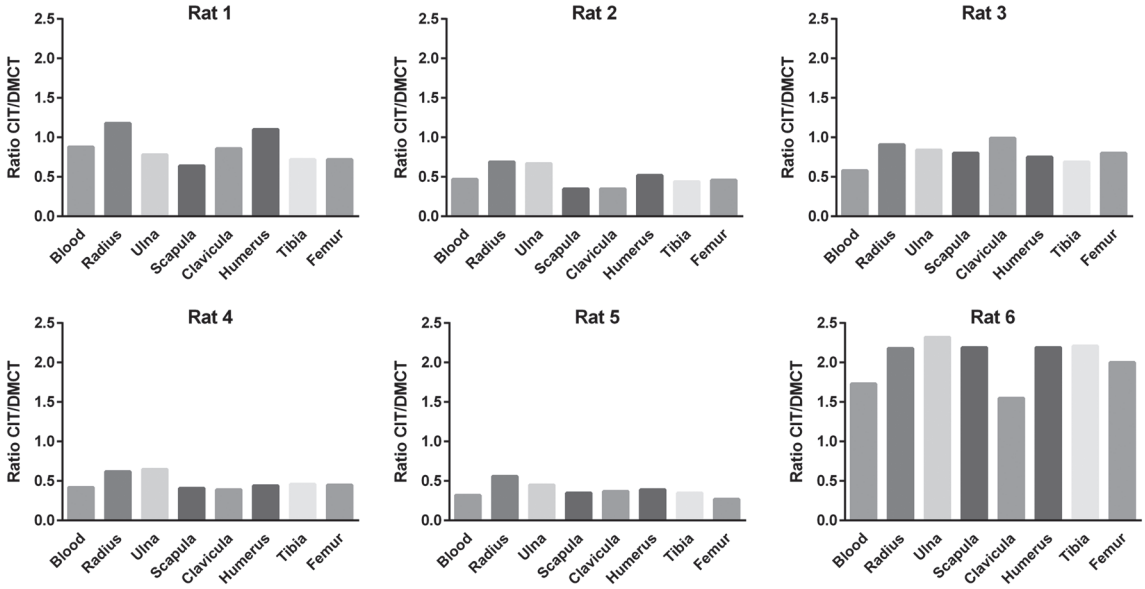


**FIGURE 5** The relative drug–metabolite ratio for citalopram/desmethylcitalopram for each bone type

of chronically dosed rats. As far as the authors know, this is the first study where chronical administration of clomipramine, citalopram, or midazolam to the rat is used, measuring both drug and metabolite levels in blood and skeletal tissue of multiple different anatomical regions 24 hours after dosing. To reach this goal, a method is fully validated according to international guidelines and the extracts of different skeletal tissues are examined at a given dose level.

In this project, an animal model was used. Therefore, the question arises whether these results are extrapolatable from rats to humans. It is known that rats are not the best model for human bone tissue, so no direct extrapolation is possible.[24] Therefore, it is also important to investigate the influences of all these factors before quantitative conclusions results can be drawn for humans. Elimination rates in rats are faster than in humans. For clomipramine and desmethylclomipramine, the half-life in rats are 7 and 5 hours, respectively.[16] Citalopram and desmethylcitalopram have a half-life of 1 and 2 hours, respectively.[25] Midazolam has an even shorter half-life of 0.5 hours.[19] Since drugs are administered for 6 months, it is safe to assume that concentrations represent steady-state values for citalopram and clomipramine.[16,19,25] Citalopram, clomipramine, and their respective metabolites are detectable in all sampled bone types. Observed skeletal tissue concentrations are comparable with those obtained from similar studies previously published and exceed those seen in blood.[10,26,27] Citalopram is found in substantially higher concentration than clomipramine. When drug concentrations of the different bone types are compared to each other within a rat, a big variability (>36%) is seen

for all drugs and metabolites. Thus, some bones are better suited for sampling than others. For citalopram and desmethylcitalopram, longer bones (humerus, tibia, and femur) result in higher concentrations compared to shorter and irregular bones (scapula, ulna, radius, and clavicula). For clomipramine and desmethylclomipramine, a similar trend is seen; however the results are not as significant. The humerus especially generally yields the highest drugs concentration for these drugs. This confirms earlier published results indicating that long bones tend to give higher concentrations.[13] The higher rate of vascularization in these bones, which poses a larger surface for drugs-bone interaction, can explain the difference.

When skeletal concentrations of drugs and metabolites in the different bone types are compared between the different rats, the variance is smaller compared to the comparison between bone types within a rat; however it is still over 28%. In our earlier published work, methadone showed concentrations found in the humeri and tibiae to be relative consistent over five rats with a relative standard deviation of less than 9%.[5] Thereby an earlier published idea was confirmed that thought skeletal tissue concentrations to be dose dependent.[13] However, this is not confirmed for clomipramine and citalopram since variability proves to be much higher. The absence of a correlation between dose and skeletal concentration can be expected since no correlation between dose and blood concentration can be found. The blood concentrations of clomipramine, citalopram, and their metabolites also show a high variance (>30%). These concentrations can be considered as therapeutically effective for depressed patients.[16] Part of the inter-individual variations in blood concentrations achieved with a given dose, can possibly be accounted for by genetic differences in the metabolism of the clomipramine and citalopram.[28]

In this study, the drug-to-metabolite relationship is also assessed. For clomipramine, approximately a 10-fold higher concentration is found compared to its more polar metabolite for skeletal tissue as for blood. For citalopram, the opposite was seen. Higher concentrations of the more polar desmethylcitalopram are found. The ratio of citalopram to desmethylcitalopram had an average of 0.86. These ratios after 24 hours are in concordance with the ratios expected to be seen in blood.[26,30] From these findings, we can assume that polarity probably does not influence the drugs disposition in bone. Therefore, more polar components are not necessary more incorporated. This indicates that probably a different mechanism is responsible for the incorporation than for example in hair tissue. For hair tissue, the suggested mechanism is passive diffusion from blood into hair follicle cells; incorporation from surrounding tissues, sweat, or sebum; and exposure to external contamination.[31]

Comparison of drugs-to-metabolite ratios in the same bone type between different rats shows a highly variable relationship for clomipramine as for citalopram. However, within a rat, these ratios are relatively consistent over all bone types with variances around and below 20% for rats dosed with clomipramine or citalopram. These ratios even show to be in close concordance with those seen in blood of the same rats. This can be an indication that the ratio is more useful for interpretation than absolute found concentration. Since the drug–metabolite ratio in bone tissue is in close concordance with the ratio seen in blood, it gives an indication of the drugs–metabolite ratio in blood at the time of death. The parent drug–metabolite ratio could give us some extra information. This can possibly help in clarifying the manner of death. It could be possible to differentiate an acute drug overdose from chronic drug use, abuse/misuse versus therapeutic drug use, or between ingestion of a parent drug and ingestion of an active metabolite that might also be prescribed.[26] Since only a small sample size is used, the significance of these quantitative skeletal drug concentrations should be interpreted with caution.

There are still a lot of gaps in bone research that need to be filled. A more precise understanding of the drug incorporation mechanism is one of them. This is critical for the proper interpretation of found drug concentration after analysis. A recent model proposes a similar model as seen in the incorporation of trace elements and stable isotopes.[8] Drugs get inside the bones through the vascular network and the channels of Haver and Volkmann. A local equilibrium will form due to ion exchange between the hydrated layer of the bone tissue and the vascular fluids. Consequently, drugs that are mostly ionized in a physiological pH (7.4) and on body temperature have a higher exchange rate and will possibly give rise to a higher concentration in this hydrated layer of bone tissue. Through substitution or chelation, these drugs will be mineralized in the crystal bone structure. This concept has already been shown in vitro for tetracycline. The model partially explains the difference in found drug concentrations within a bone and between bones. So, we can conclude that this model correlates with earlier published results that show trabecular bone to yield higher drug concentration compared to the more dense cortical bone.[5,27] This model also implies, that drug incorporation into skeletal tissue depends strongly on the drug concentration in blood, which, in turn, depends on the dosing of the drug.

Our results showed absolute skeletal concentrations to be badly correlated to blood concentrations. However, the relative drug–metabolite ratio data proved to show a better correlation between skeletal tissue and blood. This is an indication that drugs incorporation into skeletal tissue indeed depends on the concentrations found in blood. On the other side, midazolam and its metabolite showed to be absent in our extracts using this method after chronically administration. Since blood samples were taken 24 hours after the last dosing, the relative short half-life of 0.5 hours can account for the absence of midazolam in the blood. In previous studies, the concentrations of benzodiazepines found in skeletal tissue are rather low.[1,29] Therefore, a possible explanation for the absence of midazolam could be that concentrations are below our LOD (0.3 ng/g). This explanation is not likely since bone concentrations of similar molecules showed to be well above our LOD, for example diazepam (5.84 ng/g).[10] Another explanation could be found in the potential model for drugs incorporation from Rubin et al.[8] When looking at the pKa values of clomipramine (9.2) and citalopram (9.78), it is safe to assume that they are mostly ionized at physiological pH. Following the proposed model, there is a higher chance that these drugs are incorporated in bone tissue. Midazolam, however, has a pKa value of 5.5, which means it is mostly unionized at physiological pH (7.2). Therefore, the absence of

midazolam in our extracts can be explained by the possibility that it is simply not incorporated in the bone tissue. Nevertheless, midazolam and other molecules with a low pKa, like diazepam (pKa = 3.2) and colchicine (pKa = 1.85), have already been detected in bone tissue.[29-32] In the study of Gorczynski et al,[32] midazolam showed to be present in bone using an enzyme-linked immunosorbent assay (ELISA), although midazolam was also still detectable in serum. For diazepam, it is known to accumulate in bone marrow and the clearance appears to be slower in bone marrow than in blood.[33] In the study of Imfeld et al, colchicine was still present in the blood when bone concentrations were determined.[30] Bearing in mind that in bone marrow, drugs concentrations are often a delayed display of drug concentration compared to blood, there is a possibility that the concentration in bone marrow was still high. Since bone tissue consists of Haversian systems filled with vascularization, it is not impossible to imagine that drugs are not washed away in the cleaning steps because they are dried on or trapped in the interior of the bone tissue inside the channels of Haver. This could be an indication that drugs detected with pKa values under the physiological pH are not actively incorporated but can only be measured as long as they are detectable in bone marrow and hence were present in the aqueous layer of the bone tissue at the moment of death. This, in turn could mean that detection of drugs in skeletal tissue, which are not ionized under physiological pH, is an indication of relatively recent administration.

## 5 | CONCLUSION

This is the first study that has used chronically administration of clomipramine, citalopram, or midazolam to the rat, measuring both drug and metabolite in blood and skeletal tissue of multiple different anatomical regions 24 hours after dosing. An easy extraction procedure using methanol is applied and fully validated. Clomipramine, citalopram, and their respective metabolites are successfully detected and quantified in skeletal tissue of chronically dosed rats. The distribution pattern of these analytes in skeletal tissue is described. Overall, the humerus is confirmed as the best type of bone for sampling. Skeletal tissue concentrations show high variances within a rat as between rats. The variance of the drug–metabolite ratio within a rat is relatively low in the investigated bones and the ratios even shows to be in close concordance with those seen in blood. Therefore, the ratio could prove to be more useful for interpretation than absolute bone concentrations. Midazolam and its metabolite show to be absent in blood and in all bones sampled. This may implicate that compounds, which are not ionized in physiological pH, are badly or not incorporated in bone tissue. This work brings us a step closer to understanding the mechanism of drug incorporation in bone and the interpretation of found drug concentrations. In conclusion, skeletal tissue still has big potential in forensic toxicology when routine specimens are not available.

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### CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

### ORCID

*Eva Cuypers* https://orcid.org/0000-0002-6411-2461

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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