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# The effects of burial on drug detection in skeletal tissues[1]

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**Skeletal tissues have recently been investigated for use in post-mortem toxicology. Variables affecting drug concentration in these tissues, however, are still poorly characterized. In this work, the relative effects of burial on the response of enzyme-linked immunosorbent assay (ELISA) and gas chromatography-mass spectrometry (GC-MS) assays were examined. Rats were acutely exposed to ketamine or diazepam, euthanized and buried outdoors. After one month, the remains were exhumed and skeletal tissue drug levels were compared those of non-buried rats. A climate-controlled burial was also undertaken using defleshed bones to approximate an extended decomposition. Long bones (femora, tibiae) were isolated and separated into tissue type (diaphyseal bone, epiphyseal bone, and marrow), and according to treatment (i.e. buried or non-buried). Following methanolic extraction (bone) or simple homogenization (marrow), samples were analyzed with ELISA. Samples were then pooled according to treatment, extracted by solid phase extraction (SPE) and confirmed with GC-MS.**

**Under the conditions examined, the effects of burial appear to be drug and tissue dependent. Ketamine-exposed tissues demonstrated the greatest differences, especially in bone marrow. In diazepam-exposed tissues, burial did not seem to greatly affect drug response and some gave greater assay response compared to the non-buried set. Overall, the data suggest that fresh tissue samples may not be representative of decomposed samples in terms of skeletal tissue drug levels.** 

**Keywords:** forensic science; toxicology; bone; marrow; burial; ketamine; diazepam; immunoassay; gas chromatography-mass spectrometry

## Introduction

Typically, blood is the tissue of choice in post-mortem drug analysis because of the potential for correlation of drug concentrations with intoxication and fatal toxicity. Nevertheless, alternative matrices such as urine, bile, liver, hair, and muscle have been used to indicate exposure to drugs of toxicological relevance. Bone and bone marrow, on the other hand, are seldom used for toxicological analysis. However, they may be the only tissues left after a significant period of decomposition. Thus, it is important to examine the utility of these tissues to provide valuable information in post-mortem toxicology.

Although a growing number of reports are being published on the detection of drugs in skeletal tissues,[1–6] the effects of various post-mortem environments on drug concentration in skeletal tissues are still poorly understood. A handful of case studies have been presented showing the presence of drugs in skeletal tissues, most of which involve skeletonized remains and have been used to confirm the victim's identity or to corroborate a suspect's story. Amitriptyline,[7] acetaminophen and dextropropoxyphene,[8] triazolam,[9] bromisovalum,[10] methamphetamine and amphetamine[11] have been detected in bone marrow whereas aminopyrine and cyclobarbital,[12] nortriptyline,[13] and citalopram[14] have been detected in bone. These studies, however, either made use of non-decomposed tissues, or report only a particular subset of post-mortem conditions. Thus, further characterization of the implications and limitations of drug detection in skeletal tissues was warranted.

Only a small number of studies have examined the variability in assay response to bone tissue extracts after exposure to different environmental conditions. Gorczynski and Melbye examined midazolam levels in murine bone after burial in sterile and non-sterile soil and found that drug concentrations were greatest in non-buried samples, followed by samples buried in sterilized or non-sterile soil.[15] While midazolam was detected in the bone of the samples buried in the non-sterile soil, no midazolam was detected in the bone marrow of these same samples. Methamphetamine and amphetamine were measured in the marrow of rabbit bones submerged in tap water as well as from air-dried bone fragments.[16] Although amphetamine could not be measured because of interfering peaks on the gas chromatogram, methamphetamine concentrations remained constant in air-dried samples but varied in submerged samples, with no distinctive pattern over the 24-month period. Cengiz *et al*.[17] showed that morphine concentration in rabbit bone marrow decreased over time for buried samples compared to those collected immediately after death. Guillot *et al*. also examined bone marrow morphine and 6-acetylmorphine (6-AM) concentrations for acute and chronic injection of heroin.[18] After storing the bones in a jar on soil from a nearby forest for a period of two months, they saw that only 6-AM remained in the bone marrow. After the two-month storage

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period in forest soil, no drug was detectable in the bone. Finally, one author examined opiate concentrations in bone marrow of a known heroin addict and found a morphine concentration of 195 ng/g for bone marrow collected right after death.[19] Bone sampled at the same time had a morphine concentration of 340 ng/g whereas a section of this bone intentionally buried for a year had a morphine concentration of 155 ng/g, representing a 54.4% loss. Interestingly, the bone weighed 17% less after burial.

These studies suggest that post-mortem environment is a factor in observed bone drug levels, which may substantially complicate interpretation of measurements. In order to understand how this may occur, the processes associated with bone diagenesis, or changes occurring to bone in its post-mortem environment, should be considered. A variety of processes occur in bone during burial, including uptake of cations and of circulating organics, ion exchange, breakdown and leaching of collagen, microbiological attack, alteration and possible leaching of mineral matrix and infilling with mineral deposits.[20] Collagen content and porosity (which is related water uptake potential) could be postulated to influence bone drug concentration due to their effect on the exposed surface area. Collagen loss, which is mainly due to microbiological attack, contributes to weight loss (20%) and porosity increase (50%).[20]

In this work, a pilot study was undertaken in order to examine whether burial influences the response of forensic drug screening immunoassays and gas chromatography-mass spectrometry (GC-MS) confirmation assays of skeletal tissue extracts. Two drugs of forensic interest (ketamine and diazepam), selected for their varying chemical properties, were administered to rats. Drug-exposed and control animals then underwent burial, or were kept frozen until analysis. An outdoor burial and a climate-controlled indoor burial were established in order to examine different degrees of exposure to the environment and to approximate different degrees of decomposition. The goal of the work was to better understand the changes that occur in skeletal drug screening results following burial, which may assist in interpretation of the significance of drug detection in skeletal tissues.

## Materials and Methods

### Chemicals

Methanol used for drug extraction was high performance liquid chromatography (HPLC) grade and purchased from EMD Chemicals (Gibbstown, NJ, USA). Drug standards (ketamine, chlorpheniramine, diazepam, D5-diazepam, nordiazepam, and D5-nordiazepam), were obtained from Cerilliant Corp. (Round Rock, TX, USA) as 1 mg/ml methanolic solutions and diluted as required. All other chemicals were reagent grade and were obtained from EMD Chemicals (Gibbstown, NJ, USA).

### ELISA Procedure and Validation

Enzyme-linked immunosorbent assay (ELISA) analysis of bone tissue extracts was carried out as previously described[21] and used ketamine and benzodiazepine kits obtained from Immunalysis Corp. (Pomona, CA, USA). The procedure was carried out on a Chemwell 2910 Automated EIA Analyzer (Awareness Technologies, Palm City, FL, USA).

The intra-day and inter-day variation of the ELISA assay was determined for each drug. Standard solutions of ketamine and diazepam were prepared daily in PB6 (0.1 M phosphate buffer, pH=6) for use in generating standard curves to illustrate the concentration dependence for each assay. Drug concentrations in standard solutions were 5, 10, 25, 50 100 or 200 ng/mL for ketamine and from 0.5, 1, 2.5, 5, 10 and 25 ng/mL for diazepam. All standard curves included a blank, consisting of only PB6. Intra-day precision was examined by analyzing the concentration of a standard curve four times in one day. Inter-day precision was examined by making a standard curve daily and analyzing the concentration of solutions prepared, on four different days.

Matrix effects were examined by spiking drug-free skeletal tissue extracts with standard solutions of drugs at the concentrations used in generation of standard curves. Briefly, bone marrow was collected and homogenized in 0.5 M NaOH: 0.5M NaCl solution (hereafter named 50 : 50 solution) and then neutralized with acetate buffer, pH=4. Bones were cleaned in the 50 : 50 solution, rinsed in water, and dried. Bones were then ground in an all-purpose domestic grinder and incubated in methanol at 50 °C for 72 h. Methanol was then evaporated and samples were reconstituted in PBS (phosphate buffered saline). Standard solutions of drug were made in the same concentration range mentioned above in the marrow homogenate (approx. 0.1 g marrow per mL of homogenate) and in bone extracts (approx. 0.5 g bone per mL of extract) and analyzed with ELISA to examine the potential effect that bone marrow and bone matrix could have the ELISA response.

### Animal Drug Administration

All procedures for this work were approved by the Laurentian University Animal Care Committee. Adult male Wistar rats (Charles River Laboratories, Saint-Constant, Quebec, Canada) were generously donated from other researchers at Laurentian University. The rats were housed alone, in groups of two, or in groups of three, with Harlan Teklad 1/4-inch bedding (Indianapolis, IN, USA). Rats were on a 12-hour light/dark cycle, at room temperature (approximately 20 °C). They were supplied *ad libitum* with water and Harlan Teklad Laboratory Diet 8640.

Adult male Wistar rats were given 0 (n=8), 75 mg/kg (n=11) ketamine or 20 mg/kg (n=10) diazepam. All drugs were administered i.p. (intraperitoneally). Rats were sacrificed within 20 min of dosing with carbon dioxide to allow proper drug distribution, as demonstrated by previous research.[2–4] All sacrificed rats were then frozen until further treatment.

### Outdoor Burial

Drug-free (n=2), ketamine-dosed (n=3) and diazepam-dosed (n=3) rats were taken to a rural Northern Ontario site for burial. Holes, approximately two-feet deep, were dug roughly eight to ten feet apart. Rats were buried in secure wire cages and completely covered by soil. Rats were buried for a period of four weeks: ketamine-dosed rats were buried between May 28 and June 25, 2008, whereas diazepam-dosed rats were buried between August 15 and September 12, 2008. After burial, rats were exhumed and the hind legs were excised. Soft tissue was removed with a scalpel, to the greatest extent possible. All bones were frozen until further treatment. Rats that had been dosed with ketamine (n=3) or diazepam (n=2) but not buried were used to serve as a comparison (fresh samples). Drug-free rats (n=2) that were also not buried were used for each sample set to serve as negative controls.

### Climate-controlled Burial

The hind legs of sacrificed rats were removed according to the procedure described earlier. Freshly removed leg bones were buried in Schultz Moisture SoilPlus Potting Mix (Brantford, Ontario, Canada) at room temperature (approximately 22 °C). An 11.4-L receptacle was filled with two inches of soil and tibiae and femora (n=3 for each drug) were placed on top of the soil and covered by another three inches of soil. Drug-free bones were also buried to serve as a control. Bones of drug-exposed animals were frozen (n=2) to serve as a comparison. Drug-free bones (n=4) were also buried or frozen to serve as controls. After a one-month period, bones were exhumed or thawed from their vial.

### Sample Preparation - Marrow Treatment

Leg bones were separated according to bone type (femora or tibiae). Bones were cracked open with pliers to expose the medullary cavity. Bone marrow was collected with a syringe. Left and right bone marrow samples were weighed and pooled in a test tube, according to the type of bone. For animals exposed to ketamine, bone marrow was homogenized in 3 mL of 50 : 50 solution. For animals exposed to diazepam, bone marrow was homogenized in 3 mL of PB6, to minimize benzodiazepine degradation. All samples were vortexed for 10 s to ensure that all marrow was in solution. Bone marrow samples from bones buried in the climate-controlled burial were further homogenized using a rotary homogenization tool. All samples were then homogenized by ultrasonication for 30 min. Ketamine bone marrow samples were then neutralized with 3 mL of acetate buffer, pH=4.5. Samples were then diluted, if necessary, to fit into the pseudo-linear concentration range and analyzed by ELISA, according to the method described earlier.

### Bone Treatment

Bone fragments were further cracked with pliers to expose as much as the bone surface as possible. Fragments were then pooled according to their section (epiphyseal or diaphyseal section) and their type (femora or tibiae). This yielded four samples per animal. Bone fragments were submerged in the 50 : 50 solution for ketamine-exposed animals or PB8.5 (0.5 M phosphate buffer, pH=8.5) for diazepam-exposed animals. Samples were then sonicated until remaining soft tissue and bone marrow were completely removed. In the case of diazepam bones, any remaining soft tissue was cut off with a scalpel. All bone fragments were then rinsed twice with distilled water and once with methanol. Bones were then dried under a gentle stream of argon at 50 °C. Dried bones were then ground in an all-purpose domestic grinder, weighed, and placed in a screw-cap test tube. Methanol (3 mL) was accurately added to each test tube, and samples were incubated for 72 h at 50 °C. The extraction solvent was removed and bone fragments washed twice with 1 mL methanol. Washes and extract supernatatants were combined for each sample and evaporated to dryness under a gentle stream of argon at 50 °C. Samples were then reconstituted in 1 mL of PB6, and assayed by ELISA. Following the initial screen, samples were diluted to fit into the pseudo-linear concentration range of the standard curve and re-analyzed with ELISA as described earlier.

### Data Analysis

Sample response was expressed as percent decrease in absorbance, measured relative to the proper matrix-matched blank, according to the following formula:

$$\%\ Decrease\ in\ absorbance\ (\%DA) = 100\% \times \frac{(A_{ctrl} - A)}{A_{ctrl}} \qquad (1)$$

where %DA represents the percent decrease in absorbance, $A_{ctrl}$ represents the absorbance of a given matrix-matched drug-free sample, and $A$ represents the absorbance of a given sample. Values of %DA were then corrected for the dilution factor in order to evaluate ELISA response. Furthermore, samples were normalized for their mass by dividing the corrected %DA by the mass of the bone marrow or bone sample. Mean mass values were compared using Student's *t*-test, with significance attributed at $p<0.05$ (StatPlus, v.5.7.6.2). The mean %DA/mass values were also compared with the *t*-test, whereas group variability was compared using the *F*-test, with significance attributed at $p<0.05$ (Microsoft Excel 2003).

### Solid Phase Extraction

Internal standard (chlorpheniramine for ketamine sample set and diazepam-$D_5$ and nordiazepam-$D_5$ for diazepam sample set) was added to each sample. Samples were acidified with 100 µL of 7.3M $H_3PO_4$ and 3 mL acetonitrile : methanol (1:1 *v/v*) and left in the freezer overnight. Samples were then centrifuged and supernatant was removed. Samples were evaporated under a gentle stream of argon at 70 °C down to 1 mL. Acetone (2 mL) was added to each sample and left in the freezer for an hour for further precipitation. All samples were then centrifuged and supernatant was removed. Samples were evaporated down to 1 mL under a gentle stream of argon at 70 °C. Sample volume was adjusted to 5 mL with PB6 and underwent solid phase extraction (SPE).

All samples underwent extraction using Strata-XC mixed-mode columns (3cc, 60 mg, Phenomenex, Torrance, CA, USA). For ketamine extraction, columns were conditioned with 3 mL methanol, 3 mL $H_2O$ and 2 mL PB6. Samples (4.5 mL) were loaded onto the columns (0.5 mL/min), which were then washed with 3 mL 0.1M acetic acid (HOAc), 3 mL MeOH : $H_2O$ : HOAc (25 : 73:2) and 3 mL MeOH : $H_2O$ (3 : 1). Samples were then eluted with 3 mL isopropanol : $NH_4OH$ (98 : 2, 0.5 mL/min) and evaporated. For diazepam extraction, columns were conditioned with 3 mL methanol, 3 mL $H_20$ and 1 mL PB6. Samples (4.5 mL) were then loaded onto the columns (0.5 mL/min) which were then washed with 1 mL PB6, 3 mL 5% MeOH in water and 2 mL MeOH : $H_2O$ : HOAc (25 : 73:2). Samples were then eluted with 3 mL isopropanol : $NH_4OH$ (98 : 2, 0.5 mL/min) and evaporated.

### Gas Chromatography-Mass Spectrometry

Samples were analyzed with GC-MS in order to confirm the results obtained by ELISA. Samples of a given tissue and of a given treatment (i.e. buried tibial diaphyses) were pooled in order to increase the chances of detection for samples of very low concentration.

The dried residues were reconstituted in ethyl acetate (100 µL) and analyzed by GC-MS. The GC-MS used was a PerkinElmer Clarus 600 (PerkinElmer LAS, Shelton, CT, USA), equipped with a Zebron ZB-Drug-1 column (15 m × 0.25 mm × 0.25 µm, Phenomenex, Torrance, CA, USA) and operated in the constant flow mode, using electron impact ionization for ketamine and negative chemical ionization for diazepam, using ammonia as the collision gas. Helium was used as the carrier gas at a flow rate of 1 mL/min.

Injection was done in the large-volume injection (LVI) mode, and 5 µL of sample was injected. The injection port temperature was programmed, with an initial temperature of 60 °C, which was held for 3 min. The injection port temperature was then increased to 270 °C, with the split vent open and a split flow rate of 50 mL/min. The initial column temperature was 60 °C, which was held for 2 min, increased directly to 160 °C, and then increased linearly at a rate of 10 °C/min to a final temperature of 300 °C, where it was held for 3 min. Each drug was examined using selected ion monitoring (ketamine: $t_R$ 8.32 min; m/z 152, 167, **180**; chlorpheniramine: $t_R$ 9.21; m/z 167, 180, **203**; diazepam: $t_R$ 12.41 min; m/z 226, **283**, 285; diazepam-$D_5$: $t_R$ 12.38 min; m/z 231, **288**, 290; nordiazepam: $t_R$ 12.99 min; m/z **254**, 269, 270; nordiazepam-$D_5$: $t_R$ 12.99; m/z **259**, 274; ions for area comparison in bold). The response ratio was calculated as the ratio of peak area of the appropriate ions from the analyte and internal standard, where the peaks examined were required to have $t_R$ values within 1% of the expected values. The response ratio was then normalized for mass of the pooled sample. The detection limits for this method were approximately 10 ng/mL for ketamine, diazepam, and nordiazepam. Over the concentration range of 10–200 ng/mL, the precision (%CV) of replicate response ratios was less than 20% (n=3) for all standard samples assayed and the response to all analytes was linear ($R^2$ = 0.988, 0.993, 0.997, respectively).

## Results

### Concentration Dependence of the ELISA Assay

Standard solutions were made for each drug, in drug-free extracts of bone marrow or bone samples. Concentrations ranged from 0 to 200 ng/mL for ketamine and from 0 to 25 ng/mL for diazepam. Figure 1 illustrates the concentration dependence of the immunoassay. Coefficients of variation ranged from 0.17 to 18% and 0.90 to 6.2% for ketamine bone marrow and bone standards, respectively, and from 0 to 17% and 1.9 to 11% for diazepam bone marrow and bone standards, respectively.

### Physical Changes in Bones Buried in Outdoor Burial

Rats exhumed after one month of burial had undergone slight decomposition. The skin was discolored and odor of decomposition was potent. During excision of the bones, the soft tissue was removed quite easily due to the extent of decomposition. Upon examination of the bone exterior, the only observable change was an apparent reduction in soft tissue remaining on the bone itself. Upon opening of the bones, however, it was noted that the marrow had changed from a viscous dark-red substance to a less viscous, peach-brown liquid. This permitted a more complete collection of bone marrow as it did not remain on the sides of the bone in the medullary cavity.

### Effect of Outdoor Burial on Assays of Skeletal Tissue Extracts of Drug-exposed Rats

The pH of the soil was also measured, and was found to be approximately 7 for the topsoil layer, while the pH of the clay was 6.5. The mean mass of a given tissue type was compared between given treatments in order determine whether burial caused a significant reduction in tissue mass. Table 1 illustrates the differences in mass for the ketamine- and diazepam-exposed bones in the outdoor burial. Furthermore, the ELISA

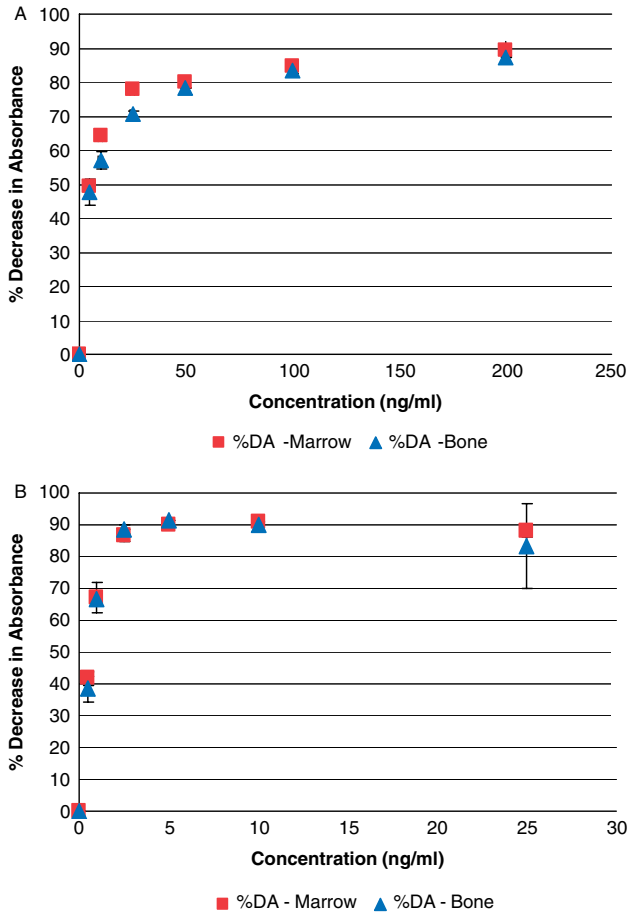


**Figure 1.** Concentration dependence ELISA response matrix matched standard solutions of A) ketamine and B) diazepam.

response (%DA) of extracts of each tissue type was examined for each treatment, after correcting for the dilution factor and the mass of tissue sampled (i.e. %DA/mass). In addition, the mean values and the variability between these treatments were examined using Student's *t*-Test and the *F*-test, respectively. Table 2 demonstrates these results for ketamine- and diazepam-exposed samples. Samples were then pooled according to their origin and treatment for further extraction and confirmation by GC-MS.

### Burial Conditions and Physical Changes in Bones Following Climate-controlled Burial

The pH of the soil used in the climate-controlled burial was measured to be approximately 7.5. While the marrow of the frozen samples was viscous and deep red in color, hardly any soft tissue remained on the epiphyses in the case of the buried bones, and soil was observed within the crevices of the epiphyseal sections. Rinsing with water was necessary to remove the soil. It was also noticed that some of the bone fragments were spotted with red coloration, which was not removed by washing. In the case of these buried bones, the bone marrow was dry and deep brown (nearly black), and scraping of the medullary cavity was necessary to remove remaining bone marrow. Additionally, a few samples also had fungus within the medullary cavity.

**Table 1.** Comparison of tissue mass for animals exposed to (A) ketamine or (B) diazepam in outdoor burial, including *p* values for *t*-test comparison of mean. Statistically significant values are bolded

**A**

| Tissue | Mean mass (g, Fresh) | Standard Deviation | Mean mass (g, Buried) | Standard Deviation | *p* (*t*-test) |
|---|---|---|---|---|---|
| Tibial marrow | 0.0206 | 0.013 | 0.0227 | 0.018 | 0.9 |
| Femoral marrow | 0.0398 | 0.019 | 0.0365 | 0.015 | 0.8 |
| Tibial diaphyses | 0.3337 | 0.047 | 0.1901 | 0.019 | **0.02** |
| Tibial epiphyses | 0.4192 | 0.054 | 0.1773 | 0.080 | **0.02** |
| Femoral diaphyses | 0.4816 | 0.132 | 0.1979 | 0.006 | 0.06 |
| Femoral epiphyses | 0.6643 | 0.037 | 0.1704 | 0.018 | **0.002** |

**B**

| Tissue | Mean mass (g, Fresh) | Standard Deviation | Mean mass (g, Buried) | Standard Deviation | *p* (*t*-test) |
|---|---|---|---|---|---|
| Tibial marrow | 0.0927 | 0.036 | 0.0294 | 0.030 | 0.2 |
| Femoral marrow | 0.1473 | 0.039 | 0.0666 | 0.031 | 0.1 |
| Tibial diaphyses | 0.7144 | 0.138 | 0.5849 | 0.093 | 0.4 |
| Tibial epiphyses | 0.7732 | 0.350 | 0.4679 | 0.039 | 0.4 |
| Femoral diaphyses | 0.8434 | 0.080 | 0.4863 | 0.132 | **0.03** |
| Femoral epiphyses | 0.8623 | 0.252 | 0.7244 | 0.057 | 0.6 |

**Table 2.** Comparison of tissue mass for animals exposed to (A) ketamine or (B) diazepam in interior burial, including *p* values for *t*-test comparison of mean. Statistically significant values are bolded

**A**

| Tissue | Mean mass (g, Fresh) | Standard Deviation | Mean mass (g, Buried) | Standard Deviation | *p* (*t*-test) |
|---|---|---|---|---|---|
| Tibial marrow | 0.0847 | 0.0005 | 0.0083 | 0.0062 | **0.002** |
| Femoral marrow | 0.1219 | 0.0072 | 0.0043 | 0.0009 | **0.03** |
| Tibial diaphyses | 0.7259 | 0.0110 | 0.6727 | 0.0713 | 0.3 |
| Tibial epiphyses | 0.4431 | 0.1138 | 0.4118 | 0.0346 | 0.8 |
| Femoral diaphyses | 0.8201 | 0.1097 | 0.6975 | 0.1819 | 0.4 |
| Femoral epiphyses | 0.7943 | 0.0997 | 0.9050 | 0.0982 | 0.3 |

**B**

| Tissue | Mean mass (g, Fresh) | Standard Deviation | Mean mass (g, Buried) | Standard Deviation | *p* (*t*-test) |
|---|---|---|---|---|---|
| Tibial marrow | 0.0556 | 0.0156 | 0.0096 | 0.0065 | 0.1 |
| Femoral marrow | 0.1158 | 0.0053 | 0.0120 | 0.0097 | **0.001** |
| Tibial diaphyses | 0.6800 | 0.0212 | 0.5859 | 0.0228 | **0.03** |
| Tibial epiphyses | 0.5430 | 0.1088 | 0.6773 | 0.1001 | 0.3 |
| Femoral diaphyses | 0.7406 | 0.0211 | 0.8002 | 0.1205 | 0.5 |
| Femoral epiphyses | 0.9155 | 0.1175 | 0.8943 | 0.0522 | 0.8 |

### Effect of Climate-controlled Burial on Assays of Skeletal Tissue Extracts of Drug-exposed Rats

The ELISA response to extracts from bone and marrow following exposure to ketamine or diazepam was examined in frozen tissues and those that underwent the climate-controlled burial. Table 2 illustrates the differences in mass for the bones in the climate-controlled burial, relative to tissues that were not buried. The ELISA response for extracts of each tissue type was examined for each treatment, after correcting for the dilution factor and the mass. Figures 2–5 illustrate these corrected results for the ketamine- and diazepam-exposed tissues.

### GC-MS confirmation

Samples of a given tissue and a given treatment were pooled for confirmation with GC-MS. Table 3 demonstrates the mass-normalized response ratio (*RR/m*) for the outdoor burials and Table 4 illustrates the *RR/m* values for the climate-controlled burials, where the response ratio represents the ratio of peak area for the analyte, relative to that of the internal standard. Total Ion Chromatograms (TICs) of the fresh (A) and buried (B) ketamine tibial bone marrow samples are shown in Figure 6, while Figure 7 illustrates a TIC for fresh (A) and buried (B) diazepam tibial bone marrow samples. Nordiazepam was also detected in the marrow samples of the frozen samples and in the tibial

**Table 3.** Mass-normalized response ratios for (A) ketamine, (B) diazepam, and (C) nordiazepam in rats buried in the outdoor burial. N/D indicates that the drug was not detected or was below the cut-off

**A**

| Tissue | RR/m (Fresh) | RR/m (Buried) |
|---|---|---|
| Tibial marrow | 9.351 | 2.849 |
| Femoral marrow | 5.900 | 5.391 |
| Tibial diaphyses | 0.017 | 0.016 |
| Tibial epiphyses | 0.093 | 0.016 |
| Femoral diaphyses | 0.005 | 0.012 |
| Femoral epiphyses | 0.047 | 0.031 |

**B**

| Tissue | RR/m (Fresh) | RR/m (Buried) |
|---|---|---|
| Tibial Marrow | 5.46 | 14.23 |
| Femoral Marrow | 3.74 | 12.60 |
| Tibial Diaphyses | 0.13 | 0.23 |
| Tibial Epiphyses | 0.98 | 1.96 |
| Femoral Diaphyses | 0.17 | 0.13 |
| Femoral Epiphyses | 1.94 | 2.39 |

**C**

| Tissue | RR/m (Fresh) | RR/m (Buried) |
|---|---|---|
| Tibial marrow | 5.41 | N/D |
| Femoral marrow | 0.409 | N/D |
| Tibial diaphyses | N/D | 0.201 |
| Tibial epiphyses | 0.123 | N/D |
| Femoral diaphyses | N/D | N/D |
| Femoral epiphyses | N/D | N/D |

marrow, femoral diaphyses and femoral epiphyses of the buried samples.

## Discussion

### Use of ELISA as an Analytical Technique for Bone and Bone Marrow Analysis

The use of small experimental animals under controlled conditions warranted the use of ELISA methods to effect the necessary sensitivity, as we have discussed previously.[3,4] Furthermore, the use of the relative decrease in absorbance (%DA) accounted, to a certain extent, for ELISA response caused by cross-reacting endogenous compounds. Any signal due to metabolites is still indicative of the presence of drugs, due in part to the fact that drug history was known. Furthermore, the presence of drug or cross-reacting metabolite was confirmed with GC-MS, although this was of limited value as samples of a given tissue type were pooled to ensure maximum probability of detection.

### Effect of Burial in an Outdoor Burial Site

Whole rats, exposed to ketamine or diazepam, were buried for one month in an outdoor setting during the summer months. The ELISA response to ketamine- and diazepam-exposed tissues was examined in bone marrow and bone samples of all buried animals,

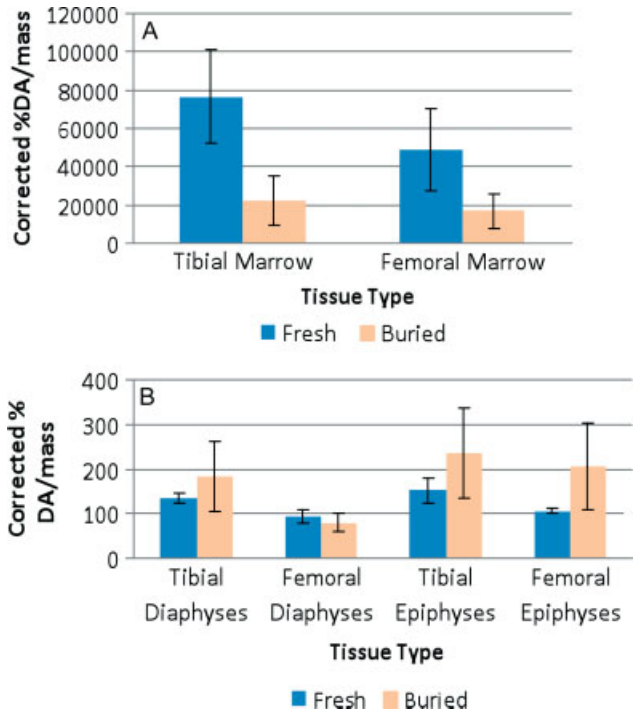


**Figure 2.** Comparison of %DA/mass of bone marrow (A) and bones (B) for ketamine-exposed rats in the outdoor burial.

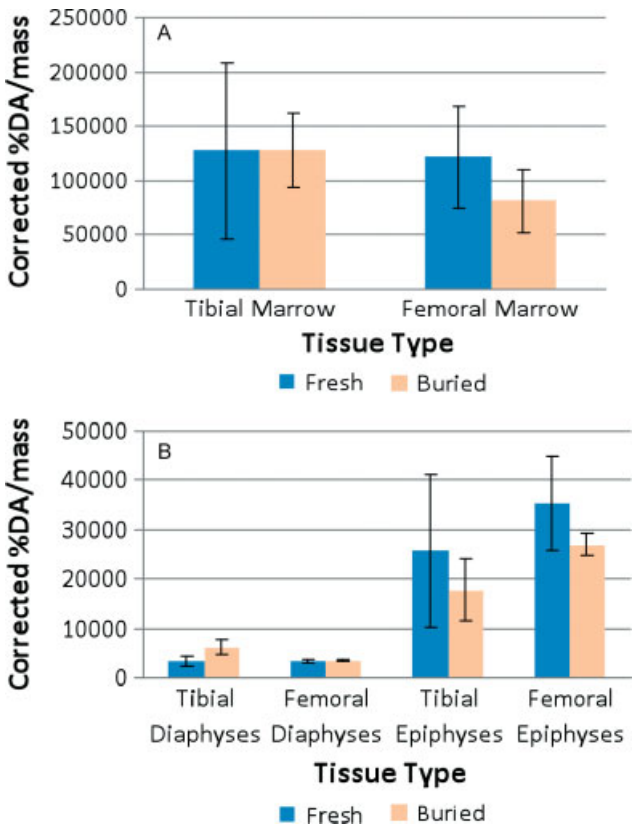


**Figure 3.** Comparison of %DA/mass of bone marrow (A) and bones (B) for diazepam-exposed rats in the outdoor burial.

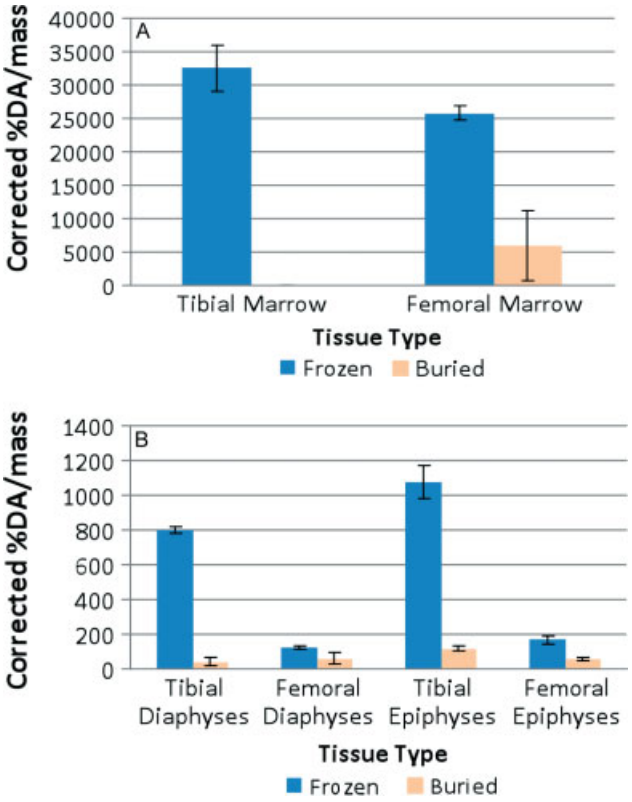


**Figure 4.** Comparison of %DA/mass of bone marrow (A) and bones (B) for ketamine-exposed rats in the interior burial.

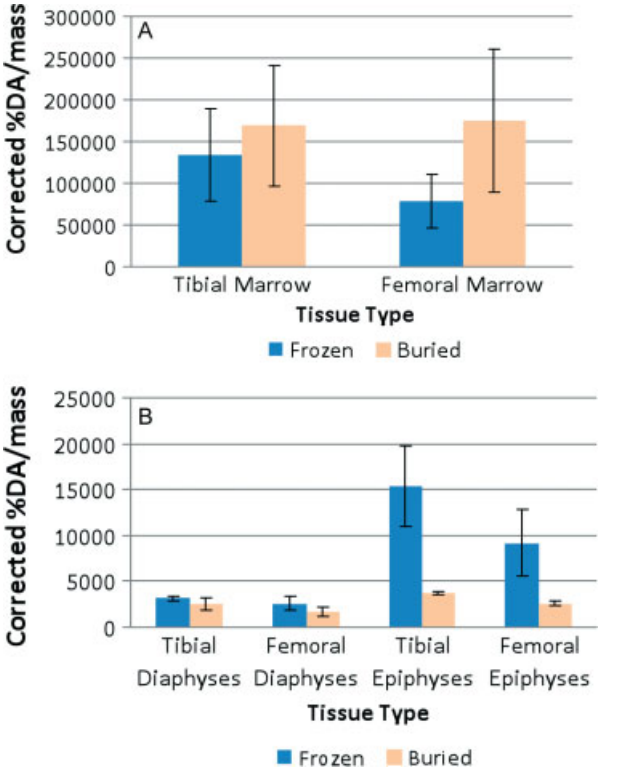


**Figure 5.** Comparison of %DA/mass of bones marrow (A) and bones (B) for diazepam-exposed rats in the interior burial.

as well as fresh samples that served as a comparison. Samples were diluted so their concentration fell within the pseudo-linear region of the standard ELISA curve to allow for comparison of %DA between samples from the different treatments. Furthermore, %DA was normalized for mass in order to account for any variation in tissue mass analyzed between samples and between treatments enabling a more accurate comparison of assay response between tissue types. Overall, there was no significant difference in mass between fresh/frozen groups and buried groups, except in the case of the tibial diaphyses, femoral diaphyses, and femoral epiphyses of the ketamine-dosed animal of the outdoor burial set.

Figure 2 illustrates the normalized %DA values for ketamine-exposed rats buried in an outdoor setting for a one-month period. Marrow samples clearly gave a greater response than bone tissues. However, statistical analysis of the %DA/mass demonstrated that only the tibial marrow of the buried animals was lower than its fresh equivalent. Furthermore, the variance appeared to differ significantly between treatments (frozen *vs* burial) for the tibial marrow, the tibial diaphyses and the femoral epiphyses. As for diazepam, the ELISA response was also greater in bone marrow than in bone (Figure 3). However, there was neither a statistical difference between any mean %DA/mass for any of the treatments, nor was there any statistically significant difference in variance between treatment groups. Overall, the ELISA data did not generally indicate a significant effect of burial in the outdoor setting. Due to the potential for cross-reactivity of metabolites and endogenous compounds, the pooled tissue samples were also analyzed by GC-MS to provide a better indication of the effect of burial on the individual drugs.

Table 3 summarizes the mass-normalized response ratio (*RR/m*) for various tissues following the outdoor burials. It should be noted again that, because the samples were pooled according to tissue type and treatment (e.g. tibial marrow/outdoor burial) following ELISA, the measured *RR/m* values represent the group average and could not undergo any statistical comparison. For ketamine-exposed tissues, the fresh samples generally gave a greater *RR/m* value, especially in the case of the tibial marrow and tibial epiphyses (Table 3A). In the case of the diazepam-exposed samples, nordiazepam was also detected in some samples. Although the %DA/mass was higher for the majority of fresh samples, these differences were not significant and the *RR/m* values determined by GC-MS results indicated a higher diazepam response in the buried samples. Nordiazepam was only detected in the fresh marrow samples, the fresh tibial epiphyses and the buried tibial diaphyses. Overall, the GC-MS data suggested a general trend of a maintenance or reduction in ketamine level and a maintenance or increase in diazepam concentration as a result of burial.

### Effect of Burial in a Climate-controlled Situation

A climate-controlled setting was utilized in an attempt to control environmental conditions and to assess the effects of 'controlled burial'. It also is a first approximation for extended decomposition in burial since overlying soft tissue was removed. Significant physical changes were noticed in the buried bones, where marrow in the medullary cavity was completely dry. Marrow removal was accomplished by scratching the sides of the bone with the syringe needle. Furthermore, bone marrow of dried samples needed to be homogenized further by manual homogenization with a rotary tool.

As was seen in the outdoor burial, the marrow of the fresh ketamine-exposed rat bones gave higher %DA/mass than the

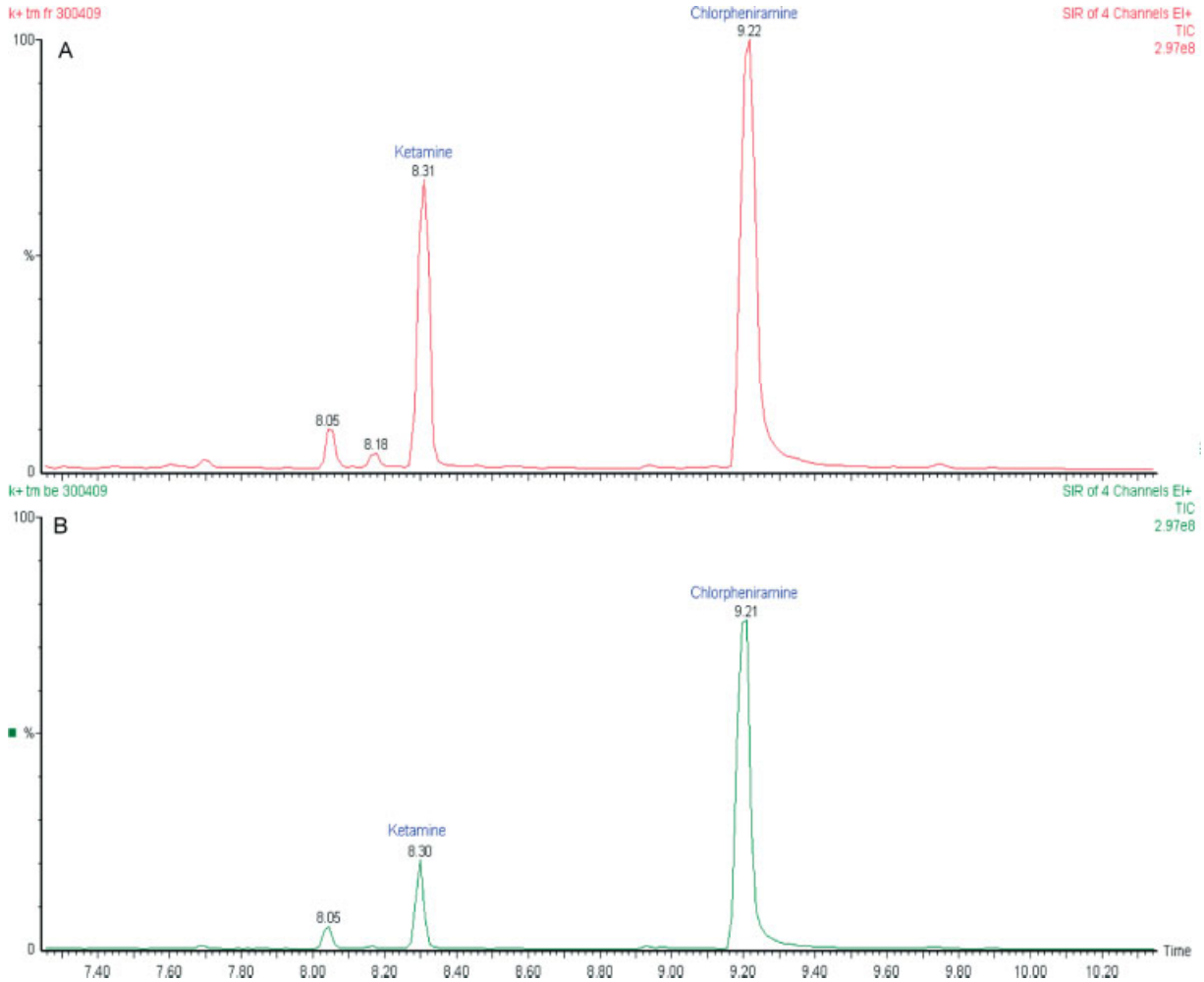


**Figure 6.** TIC for fresh tibial marrow (A) and buried tibial marrow (B) for ketamine-exposed rats in the outdoor burial.

buried counterpart, as did the fresh tibial fragments relative to the buried tibial fragments (Figure 4 and Table 5). However, no such difference was noticed in the femoral fragments. In the case of the diazepam-exposed samples, the marrow of the buried samples actually gave a higher ELISA response (Figure 5) than the frozen samples, but the difference was not significant. Surprisingly, the variance of both the tibial epiphyses and the femoral epiphyses were higher for the frozen samples than for the buried samples (Figure 5 and Table 5). Overall, the corrected ELISA response remained unchanged or declined following indoor burial of ketamine-exposed tissues, unchanged or increased in marrow extracts of diazepam-exposed animals, but unchanged or declined in bone extracts of diazepam-exposed animals.

Table 4 illustrates the mass-normalized response ratio (*RR/m*) values for tissue extracts from the interior burial. As was seen with ELISA data, the frozen ketamine-exposed samples had a much higher response; ketamine was not detected in buried samples by GC-MS. In extracts of the frozen tissues, ketamine was detected only in the femoral marrow, as well as in tibial and femoral epiphyses (Table 4A). In the case of the diazepam-exposed tissues, diazepam was detected in all samples in the interior burial (Table 4B). Although the frozen bone samples had a higher %DA/mass with ELISA, the bone tissues from the buried samples had higher *RR/m* values. Nordiazepam was detected in the marrow samples of the frozen samples and in the tibial marrow, femoral diaphyses, and femoral epiphyses of the buried samples. There were no trends in the measured *RR/m* values for nordiazepam in the frozen and buried tissues that would corroborate the ELISA data, so it may be that there was the presence of a cross-reactant, potentially another diazepam metabolite, in the frozen tissue samples that was not present in the buried samples.

### Implications of Findings for Interpretation of Drug Measurements in Skeletal Tissues

Overall, it appears that, under the conditions examined, the effects of burial may be drug dependent and that marrow drug levels may exhibit more noticeable changes due to burial. Significant changes in %DA/mass after burial were noticed more frequently in samples of ketamine-dosed rats (Table 5), and were associated with reductions in ketamine assay response following burial. Also, the variance for a given treatment also varied with drug exposure: ketamine-exposed tissues showed a greater variance in %DA/mass due to burial in the outdoor burial (Table 5). Since both ketamine and diazepam are known to be relatively stable drugs,[22,23] it is not sufficient to suggest that drug losses are due to degradation only. Further, nordiazepam has been reported to be unstable in post-mortem tissues.[24] Since benzodiazepine ELISA responses tended to increase, as did *RR/m* values for diazepam (and, in some

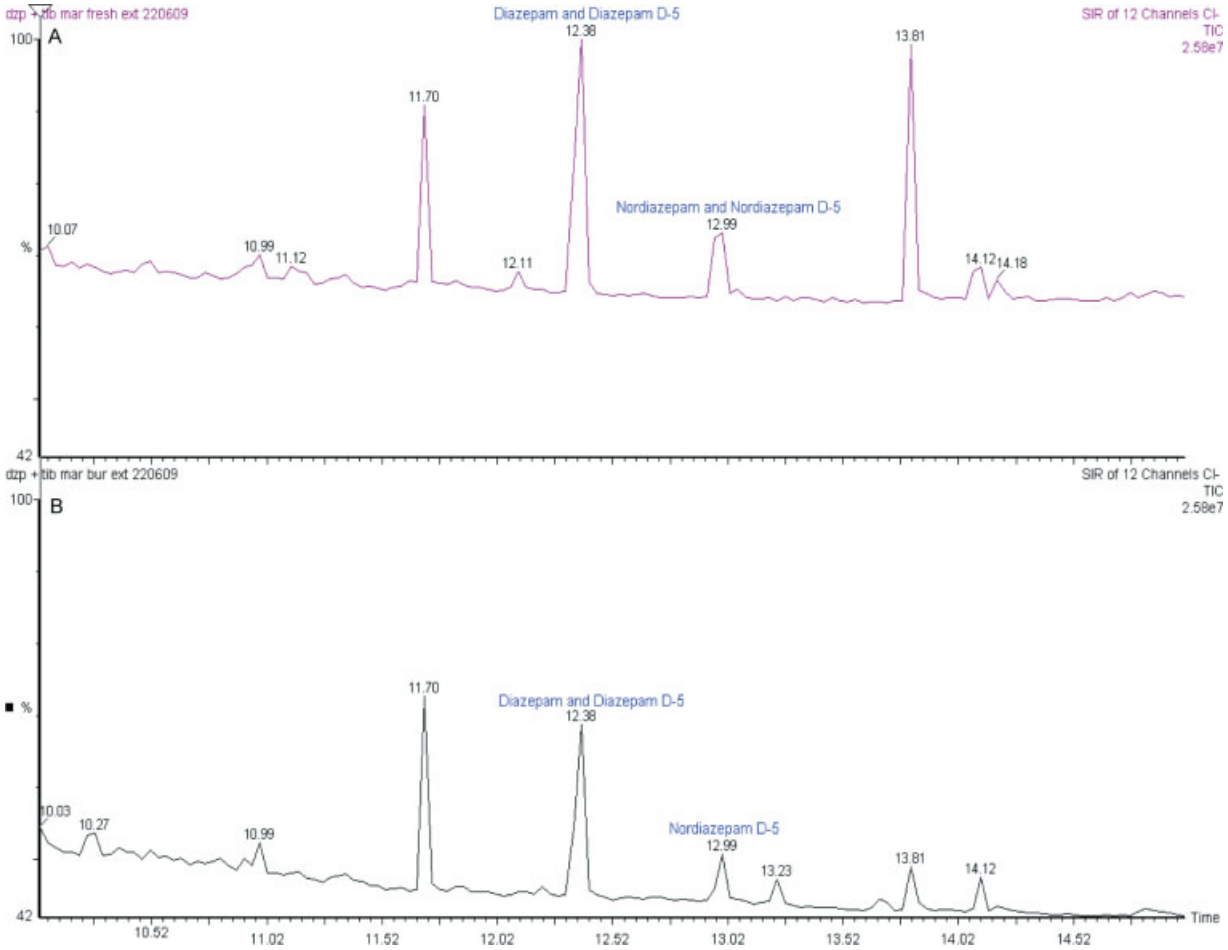


**Figure 7.** TIC for fresh tibial marrow (A) and buried tibial marrow (B) for diazepam-exposed rats in the outdoor burial.

cases, for nordiazepam) following burial, one potential factor in the observed differences is changes in drug concentration as the viscous marrow decomposed into a more fluid product. Differences in water solubility may result in either concentration of less water-soluble drugs or losses of more water-soluble drugs as the fluid matrix flows out of the bone sample.

These results may have important ramifications on the possibility to quantitatively interpret toxicological data from decomposed bone and bone marrow samples, in that they suggest that it is inherently difficult to find an appropriate reference matrix condition. In other words, the use of a database of bone drug levels obtained from fresh bone only may not be representative of levels obtained from decomposed bones, even if the initial drug exposure conditions were similar. The body of literature that exists with respect to drug levels in human bones is somewhat disjointed, with most larger-scale studies[1,6] making reference largely to only relatively fresh tissues, with individual case reports more often reporting levels in decomposed tissues.[7–11] Thus, at the present time, it seems more prudent to present toxicological findings in a qualitative manner (i.e. detected or not detected) in forensic casework.

### Implications and Limitations of Analytical Methodology

While the *t*-test was used in order to compare the mean values for tissue mass and normalized ELISA response, the trends observed should be re-examined in a larger study, including a wider range of decomposition conditions and drug exposures, and with a larger sample size. Furthermore, although the Shapiro-Wilk W normality test suggested that all sample sets possessed a normal distribution, with the exception of the frozen diaphyses and the buried marrow for the ketamine-exposed samples of the climate-controlled burial and the buried marrow of the diazepam-exposed samples of the climate-controlled burial, the *F*-test is susceptible to effects of deviations from normality. The significance of the variance comparisons for these samples should be taken with some reservation, but the notion of significant changes in sample variance with changes in decompositional state merits further consideration in future work.

Following ELISA, samples of a given tissue type from animals of a given treatment group (i.e. buried *vs* non-buried) were pooled prior to analysis by GC-MS in order to increase the probability of detection. The GC-MS results indicated that detection of ketamine and diazepam was still possible after burial for a period of one month and was not simply an endogenous compound that cross-reacts with the ELISA assay. Although some of the GC-MS results differed from those found with ELISA, it may be due in part with the way the samples were analyzed (pooled samples for GC-MS *vs* individual analysis for ELISA), as well as the potential for cross-reactivity to metabolites and other compounds in ELISA measurements. Despite the potential for cross-reactivity, ELISA remains a very sensitive technique for measurements in experimental studies of drug disposition in bone tissue. In the

**Table 4.** Mass-normalized response ratios for (A) ketamine, (B) diazepam and (C) nordiazepam of bones buried in the interior burial setting. N/D indicates that the drug was not detected or was below the cut-off

**A**

| Tissue | RR/m (Fresh) | RR/m (Buried) |
|---|---|---|
| Tibial marrow | 1.46 | N/D |
| Femoral marrow | 2.23 | N/D |
| Tibial diaphyses | 0.02 | N/D |
| Tibial epiphyses | 0.10 | 0.02 |
| Femoral diaphyses | N/D | N/D |
| Femoral epiphyses | N/D | N/D |

**B**

| Tissue | RR/m (Fresh) | RR/m (Buried) |
|---|---|---|
| Tibial marrow | 23.70 | 53.95 |
| Femoral marrow | 10.53 | 18.34 |
| Tibial diaphyses | 0.19 | 0.28 |
| Tibial epiphyses | 1.00 | 1.12 |
| Femoral diaphyses | 0.33 | 0.48 |
| Femoral epiphyses | 1.07 | 0.58 |

**C**

| Tissue | RR/m (Fresh) | RR/m (Buried) |
|---|---|---|
| Tibial marrow | 9.00 | 13.52 |
| Femoral marrow | 1.30 | N/D |
| Tibial diaphyses | N/D | N/D |
| Tibial epiphyses | N/D | N/D |
| Femoral diaphyses | N/D | 0.07 |
| Femoral epiphyses | N/D | 0.09 |

**Table 5.** *p* values for comparisons of %DA/mass values for tissues with significantly different means (*t*-test) and significantly difference variances (*F*-test)

| Tissue type | *p* value (*t*-test) | *p* value (F-test) |
|---|---|---|
| Tibial marrow – ketamine outdoor burial | **0.04** | **0.04** |
| Tibial diaphyses – ketamine outdoor burial | N/A | **0.04** |
| Femoral epiphyses – ketamine outdoor burial | N/A | **0.01** |
| Tibial marrow – ketamine interior burial | **0.04(7)** | N/A |
| Femoral marrow – ketamine interior burial | **0.02** | N/A |
| Tibial diaphyses – ketamine interior burial | **0.0001** | N/A |
| Tibial epiphyses – ketamine interior burial | **0.04** | N/A |
| Tibial epiphyses – diazepam interior burial | N/A | **0.008** |
| Femoral epiphyses – diazepam interior burial | N/A | **0.01** |

rat tissues examined here, the mass of a given bone sample was always below 1 g. In human bone samples, samples would probably exceed this mass and a greater amount of bone could be extracted and analyzed. Therefore, samples analyzed in human decomposition cases may have a greater probability of detection using conventional techniques such as GC-MS and LC/MS/MS, as has been demonstrated previously.[1,6]

It was clear from these data that the two modes of burial examined were not equivalent in terms of the effect on tissues. This is not surprising given that the indoor burial did not involve the normal temperature fluctuations of the natural environment, and there was a total absence of insect activity. Further, the indoor burial examined bones that had been previously defleshed. While this serves as one approximation of an extended decomposition period, it does not account for changes to bone tissues that may occur while soft tissue decomposition occurs. This approach, however, did provide the opportunity to make qualitative observations and some measurements of the changes in bone tissue under different burial conditions. The bone marrow of the samples buried in the climate-controlled situation were dry and, more often than not, lighter in mass than the frozen samples; whereas the bone marrow of the samples buried in the outdoor burial remained fluid and tended to be similar in mass to the fresh samples (Tables 1 and 3). Furthermore, statistically significant changes in mean values and variance of immunoassay response were different in the two burial situations for a given drug. Most effects on immunoassay response associated with burial were also observed in the GC-MS analysis of pooled samples.

It is not possible with this data to determine what may be causing differences in assay response as a result of burial, and why those effects appeared to be at least somewhat drug dependent in this study. It remains to be seen whether these findings would be replicated in a larger-scale study of various burial situations. Again, given the paucity of information on post-mortem changes on drug disposition and concentrations in skeletal tissues, the ability to quantitatively report and interpret drug concentrations in bones may not be appropriate. In forensic casework, it may be more reasonable to report skeletal tissue drug concentrations in terms of qualitative results (i.e. detected or not detected).

Examination of the effects of burial on skeletal tissues revealed that it may not be possible to extrapolate data from fresh skeletal tissue samples to buried and decomposed skeletal tissue samples, and that the mode of decomposition may effect the degree of deviation of data from those derived from fresh tissues. Assay response in both ELISA and GC-MS assays to extracts of fresh tissue samples may differ from those of extracts of buried tissues. Therefore, it appears that interpretation of data regarding fresh tissues may be limited in terms of forensic utility. The use of fresh samples, however, does permit more controlled experimentation. This type of analysis has shown to be useful for the method development and optimization of drug detection from skeletal tissues. Thus, analysis of fresh skeletal tissues remains an integral part of understanding principles of drug disposition in skeletal tissues.

More research in the interpretive value of drug measurements in skeletal tissues is needed. Important variables that still require investigation are decomposition interval, post-mortem environment (water submersion, cremation, and surface decomposition), and pattern of drug administration (e.g. acute, chronic, fatal overdose, delayed death). Given that some changes in drug level do appear to occur during decomposition processes, it may be valuable to examine the ratio of parent and metabolite concentrations in order to determine if these parameters may be less prone to the

effects of tissue change related to decomposition. Within this area, an examination of chronic dosing would be necessary, to examine if chronic dosing would yield different distribution patterns within the skeletal tissues.

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