Relative Distribution of Drugs in Decomposed Skeletal Tissue

James H. Watterson[1], Nathalie A. Desrosiers[1], Caroline C. Betit[1], Dorothy Dean[2], and **John F. Wyman[3]**

[1]Forensic Toxicology Research Laboratory, Laurentian University, 935 Ramsey Lake Rd., Sudbury, Ontario, Canada P3E 2C6; [2]Summit County Medical Examiner's Office, 85 North Summit Street, Akron, Ohio 44308; and [3]Franklin County Coroner's Office, 520 King Avenue, Columbus, Ohio 43201

Abstract

Skeletal tissues from a domestic pig exposed to amitriptyline, diazepam, and pentobarbital were analyzed to determine the relative distribution of these drugs in bone. Following drug exposure and euthanasia, remains were allowed to decompose outdoors to complete skeletonization between summer 2007 and fall 2009. Remains were recovered and separated according to bone type. Twelve different bone types were pulverized and sampled in triplicate. Each bone sample underwent methanolic extraction (96 h, 50°C), followed by solid-phase extraction and gas chromatography–mass spectrometry in the selected ion monitoring mode. Mass-normalized assay responses underwent ANOVA with post-hoc testing, revealing bone type as a main effect for all three drugs, but not for the diazepam metabolite (nordiazepam). The assay response varied with respect to bone type by factors of 27, 39, and 20 for pentobarbital, diazepam, and amitriptyline, respectively. The relative distribution between bone type was qualitatively similar for the three administered drugs analyzed for, with the largest response obtained from rib for all three drugs. This is the first study, to the authors' knowledge, of the distribution of different drugs in various decomposed skeletal tissues in a controlled experiment using an animal model of comparable physiology to humans. These data have implications for the interpretive value of forensic drug measurements in skeletal tissues.

Introduction

In the last decade, there have been a number of reports describing analysis of drugs in skeletal tissues (1–10). A variety of circumstances were described in these reports, including studies using postmortem human tissues (1,3), case reports (2,4), and basic research using experimental animals under controlled conditions of dose, delay between administration and death, and the degree of decomposition (5–10). Within these studies, there have been a variety of different skeletal tissues sampled, and a number of different methods used in the isolation of drugs from the solid bone matrix and subsequent extraction of drugs from these preliminary extracts. A common occurrence within these reports is the use of only a small number of different skeletal tissues. Among those examined have been diaphyseal femoral bone (1), iliac bone (2,3), vertebral bone and pelvic bone (6), as well as marrow from various sources (4–10). Although it is certainly clear that drug concentrations in marrow may be substantially greater than those in bone, there remain very little data describing the relative distribution of different drugs in different bones. Ideally, these data should be collected using bone tissues that have undergone substantial decomposition, as this may be the primary situation in which bone may be a likely candidate for analysis (i.e., when blood and conventional visceral tissues are not available). Furthermore, the use of experimental animals such as rats provide some value in terms of being able to control experimental variables such as drug use history, route of administration and post-administration/postmortem circumstances, but are limited in terms of direct applicability to the human case. When using rats as an experimental model, the analysis typically requires analysis of a proportionally large fraction of a particular tissue type from each animal. For example, analysis of femoral bone tissues in a rat may involve analysis of most or all of the marrow from each femur, and the entire diaphyseal or epiphyseal fragment, in order to sample a sufficient tissue mass for drug detection by conventional analytical methods such as immunoassay or gas chromatography–mass spectrometry (GC–MS) (5–8). This represents a situation that may be inconvenient or impossible for a forensic laboratory to undertake in casework. It remains unclear whether one bone type may be more advantageous for sampling to establish drug exposure and, by extension, whether a particular bone section may be the best choice.

In this work, we report the results of analysis of the skeletal remnants of an adolescent domestic pig (*Sus domesticus*) that received a mixture of drugs (amitriptyline, citalopram, di-

azepam, and morphine) by acute oral administration prior to euthanasia by an intracardiac overdose of pentobarbital. This pig was used in studies conducted in the summer of 2007 wherein a number of pigs received a variety of different drug cocktails in order to examine drug stability in decomposing tissues (11), and skeletal remains were recovered from the site of this experimentation in November 2009, with no soft tissue left on any of the bones recovered. A variety of skeletal tissues were analyzed for the presence of amitriptyline, diazepam (as well as the metabolite, nordiazepam), and pentobarbital, based on methods currently in use in our laboratory, such that the relative distribution of each drug or metabolite could be assessed in a semi-quantitative fashion, in order to address the question of whether toxicological measurements in one skeletal tissue fragment are representative of those from another anatomic site. One valuable aspect of examining skeletal tissues for these particular drugs is the wide variability in terms of chemical properties, including a basic compound with high volume of distribution (amitriptyline), a very weakly basic/neutral compound with moderate volume of distribution (diazepam), and a weakly acidic compound with low volume of distribution (pentobarbital).

Methods

Chemicals

Methanol used in drug extraction was high-performance liquid chromatography grade and purchased from EMD Chemicals (Gibbstown, NJ). Drug standards (Cerilliant, Round Rock, TX) were obtained as 1 mg/mL methanolic solutions and diluted as required. All other chemicals were reagent grade and obtained from EMD Chemicals. Trimethylphenyl ammonium hydroxide (TMPAH), used as an alkylating reagent in the derivatization of barbiturates, was obtained from United Chemical Technologies (Bristol, PA).

Animals

The skeletal remains examined were derived from a single skeleton of an animal which was part of the set of animals used in a study of the temporal fate of drugs in postmortem tissue published elsewhere (11). The animals used in that study were a Yorkshire/Hampshire cross-breed, females and/or male castrates (as available), and weighed approximately 120 to 180 pounds (55 to 82 kg). Pigs were obtained from Kidron Auction (Kidron, Ohio). To administer drugs, pigs were restrained using a nose snare and with the aid of a speculum, drug cocktails were placed in the stomach by gavage using a 500 mL syringe and gastric tube. The current work concerns the skeletal remains of one of the animals from the group given a cocktail containing amitriptyline (75 mg/kg), citalopram (7 mg/kg), diazepam (7.5 mg/kg), and morphine (0.8 mg/kg) were analyzed. Four hours after dosing, animals were anesthetized (30 mg/kg pentobarbital IP) and then sacrificed while sedated by intracardiac injection of 10 mL Beuthanasia-D® [(390 mg sodium pentobarbital and sodium phenytoin, 390 and 50 mg/mL, respectively) Schering-Plough Animal Health, Union, NJ] solution using an 18G × 3½ spinal needle (BD Medical Systems). The original study was performed in accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals", and it was approved by the ILACUC, The Ohio State University, Columbus, OH.

Sample preparation

Samples were prepared for analysis for amitriptyline, diazepam, nordiazepam, and pentobarbital, based on methods currently in use in the laboratory of some of the authors (Watterson, Desrosiers, Betit). Bones were first separated according to anatomic location (e.g., ribs, scapulae, vertebrae, pelvi) and, in the cases of humeri, tibiae, and femora, separated into diaphyseal and epiphyseal fragments. Bones were rinsed twice in distilled water, followed by acetone and then dried. Bones were initially crushed manually and then ground to a fine powder using a domestic grinder. Samples (n = 3) of pulverized bone (3 g) from each bone type were accurately weighed and incubated in 10 mL methanol in screw-cap tubes for 96 h. Drug-free porcine bone, donated by the Forensic Osteology Laboratory at Laurentian University, was used as a negative control. Each tube underwent vigorous agitation by vortex periodically throughout the incubation period. For each sample, methanolic supernatants were recovered and the bone matrix was washed with 5

Table I. GC–MS Parameters for Analysis of Various Analytes†

Analyte	Initial Column Temperature (Hold Time)	Temperature Ramp (Final Column Temperature)	Final Column Hold Temperature (Hold Time)
Amitriptyline (189, 202.2, 215; 219*, 228*, 268*)	60°C (3 min)	100°C/min (160°C; hold 1 min) 12°C/min (300°C)	300°C (3 min)
Diazepam/Nordiazepam) (221, 256, 283; 239, 242, 270; 226*, 261*, 288*)	150°C (3 min)	100°C/min (160°C; hold 1 min) 10°C/min (300°C)	300°C (3 min)
Pentobarbital (113, 169, 184; 181*, 195*, 196*)	60°C (3 min)	100°C/min (160°C; hold 0 min) 10°C/min (220°C; hold 0 min) 100°C/min (300°C)	300°C (3 min)

† Ions used for quantitative analysis are underlined. Ions corresponding to the internal standard are marked with an asterisk.

mL clean methanol, with vigorous agitation. Each methanol wash was combined with the corresponding supernatant and then evaporated to dryness under a gentle stream of air. Residues were reconstituted in 2 mL 0.1 M phosphate buffer (PB6, pH 6) and acidified with 100 mL glacial acetic acid. An internal standard (ITSD) solution (25 µL) containing secobarbital, clomipramine, and diazepam-d_5 was added to each sample such that the final concentrations of each internal standard were 250, 250, and 100 ng/mL, respectively. Each sample then underwent precipitation of lipids and proteins by addition of cold acetonitrile/methanol (1:1, v/v, 3 mL), followed by storage at –20°C for 1 h, and centrifugation (3800 rpm, 10 min). Supernatants were isolated and evaporated to approximately 2 mL (PB6 fraction). The solution volume was then adjusted to 5 mL with PB6 and each sample underwent further purification by solid-phase extraction (SPE).

SPE was done using Clean Screen CSDAU columns (3 cc, 200 mg, United Chemical Technologies, Bristol, PA) for amitriptyline and pentobarbital. Although diazepam and nordiazepam were also extracted using these columns, a coeluting matrix component interfered with measurements of the internal standard used for these drugs. Consequently, Clean Screen CSBNZ columns (3 cc, 200 mg, United Chemical Technologies) were used in a separate extraction for diazepam and nordiazepam. Because of sample limitations, fibulae and scapulae could not be included in that sample set. Initial steps of conditioning, loading and washing were done on a Gilson ASPEC GX-724 automated SPE instrument (Gilson, Middleton, WI). CSDAU columns were conditioned with 3 mL of methanol and deionized water, followed by 2 mL PB6. Samples were loaded (4500 µL) at a rate of 0.5 mL/min. Columns were then washed sequentially with PB6 (2 mL), 0.1 M acetic acid (2 mL), and 3% acetic acid in 25:75 methanol/deionized water (3 mL). Columns were then transferred to a vacuum manifold and dried for 5 min at 10 in. Hg. Columns then underwent a final wash step consisting of two column volumes (approximately 3.5 mL each) of hexane, followed by drying for 5 min at 10 in. Hg. Drugs were eluted with two column volumes of 2% NH_4OH in 80:20 ethyl acetate/isopropanol (v/v). CSBNZ columns were conditioned with 3 mL of methanol and deionized water, followed by 2 mL PB6. Samples were loaded (4500 µL) at a rate of 0.5 mL/min. Columns were then washed sequentially with PB6 (2 mL) and 10% (v/v) acetonitrile in PB6 (3 mL). Columns were then transferred to a vacuum manifold and dried for 5 min at 10 in Hg. Columns then underwent a final wash step consisting of two column volumes (approximately 3.5 mL each) of hexane, followed by drying for 5 min at 10 in Hg. Drugs were eluted with two column volumes of 2% NH_4OH in 80:20 ethyl acetate/isopropanol (v/v). All extracts were then evaporated to dryness under a gentle stream of air (70°C) and reconstituted in 100 µL ethyl acetate for analysis by gas chromatography–mass spectrometry (GC–MS).

GC–MS

Extracts were analyzed on a PerkinElmer Clarus 600C GC–MS (PerkinElmer LAS, Shelton, CT) using electron impact ionization in the selected ion monitoring (SIM) mode. The MS source and transfer line temperatures were 250°C. The electron energy used was 70 eV. Extracts (2 µL) were injected into the injector maintained in splitless mode at 250°C. Analyses of amitriptyline, diazepam/nordiazepam, and pentobarbital were done in three sequential injections, using different temperature ramps. The ions monitored and column temperature conditions used in each analysis are summarized in Table I. Prior to injection of the sample for pentobarbital analysis, 100 µL trimethylphenyl ammonium hydroxide (TMPAH, United Chemical Technologies) was added to each autosampler vial, for purposes of flash methylation of the barbiturate analytes. Criteria for positive analyte detection included agreement of analyte relative retention time to within three standard deviations of standard samples, and agreement of peak-area ratios of quantitative to qualifier ions for each analyte to within 20% of standard samples. The analytical parameter used in all measurements was the mass-normalized response ratio (RR/m), or

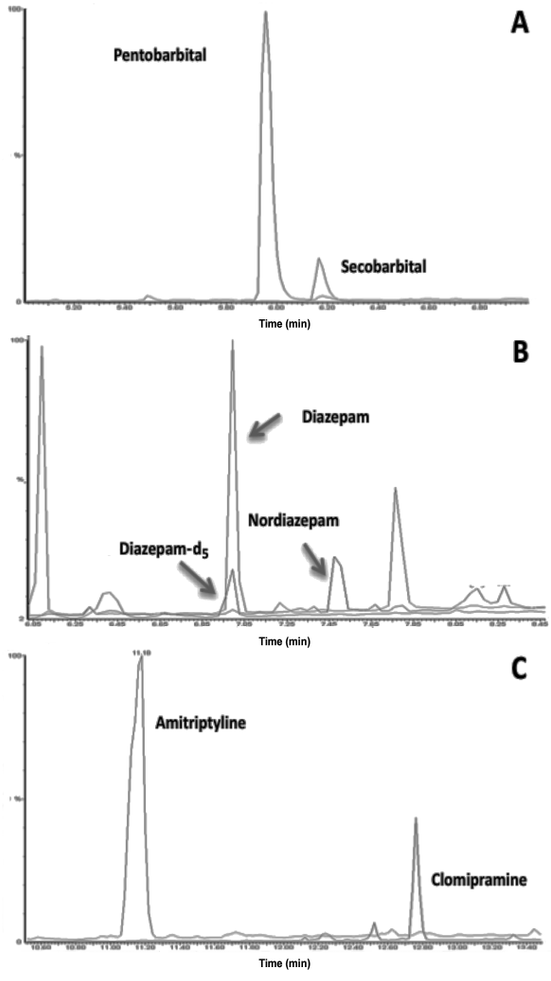

Figure 1. SIM chromatogram of analysis of pentobarbital (secobarbital as internal standard) in extract of femoral epiphyseal bone (A); SIM chromatogram of analysis of diazepam and nordiazepam (diazepam-d_5 as internal standard) in extract of rib bone (B); and SIM chromatogram of analysis of amitriptyline (clomipramine as internal standard) in extract of vertebral bone (C).

ratio of peak area of analyte to that of ITSD, divided by the mass of tissue sampled.

Extracted standards showed a linear relationship between response ratio and analyte concentration, with correlation coefficient (R^2) values of 0.998, 0.978, 0.993, and 0.998 for amitriptyline, diazepam, nordiazepam, and pentobarbital, respectively. Precision, measured as the coefficient of variation [CV(%)], of replicate analyses of extracted standards was within ± 20% for samples over the range of 10–500 ng/mL for each of these analytes, with the exception of diazepam, for which CV(%) values reached 30% at 25 ng/mL. Linearity of the analytical method over an extended concentration range (up to 4000 ng/mL) was verified for pentobarbital and amitriptyline (R^2 = 0.999 and 0.995, respectively), as the measured mass-normalized response ratios exceeded those of standards at concentrations typically observed in our laboratory analysis of drugs in bone tissue (i.e., less than 500 ng/mL).

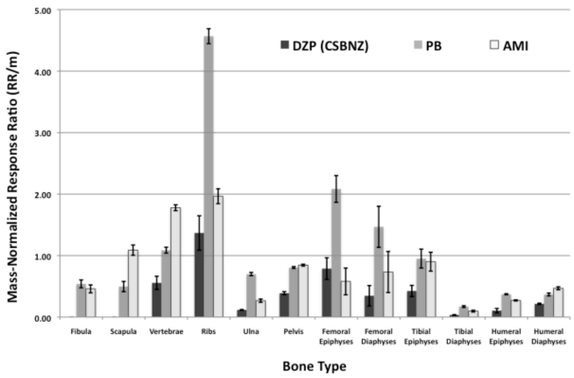

Figure 2. Mean mass-normalized response ratio (RR/m) values (error bars represent 1 SD) for analyses of amitriptyline (AMI), diazepam (DZP), and pentobarbital (PB) from extracts of various bone types.

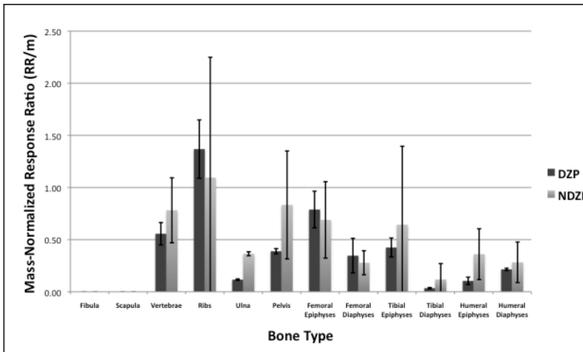

Figure 3. Mean mass-normalized response ratio (RR/m) values (error bars represent 1 SD) for analyses of diazepam (DZP) and nordiazepam (NDZP) from extracts of various bone types.

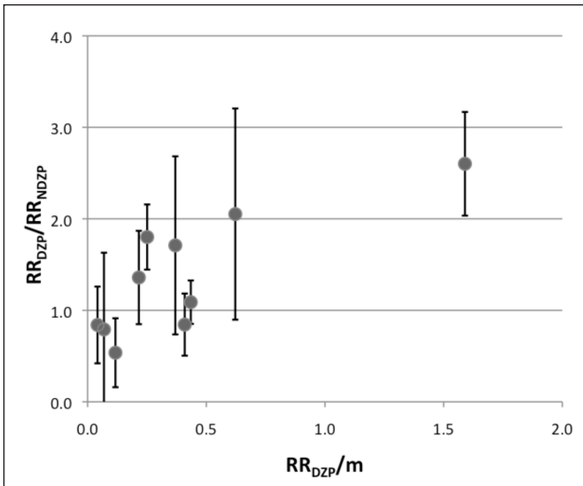

Figure 4. Plot of the ratio of mass-normalized assay responses (i.e., $(RR/m)_{DZP}/(RR/m)_{NDZP}$) for diazepam and nordiazepam as a function of $(RR/m)_{DZP}$.

Statistics

The set of all replicate measurements of RR/m for each bone type were analyzed using ANOVA (one-way) with Tukey's post-hoc analysis to assess differences between group means. Statistical analysis of the data was done using StatPlus software (v. 5.7.6.2, AnalystSoft).

Results

All GC–MS data are expressed in terms of the analyte/ITSD response ratio, normalized for the mass of bone sampled (i.e., RR/m). Example SIM chromatograms for the analysis of amitriptyline, diazepam/nordiazepam, and pentobarbital from various bones types are shown in Figure 1. Mean RR/m values, along with standard deviations (SD) are plotted for all drugs administered against bone type in Figure 2, and for diazepam and nordiazepam in Figure 3. Coefficients of variation (CV%) for replicate extractions (n = 3) of the various bone types ranged from 3 to 21%, 5 to 47%, and 2 to 23% for amitriptyline, diazepam, and pentobarbital, respectively. The measured RR/m values correspond to extracted drug concentrations ranging from approximately 30 to 1650 ng/g for amitriptyline, 50 to 205 ng/g for diazepam (excluding the ulnar, humeral diaphyseal, humeral epiphyseal, and tibial diaphyseal samples, in which the diazepam was below the limit of quantitation, 25 ng/mL), 40 to 130 ng/g for nordiazepam (excluding the tibial diaphyseal samples, in which the diazepam was below the limit of quantitation, 25 ng/mL), and 30 to 630 ng/g for pentobarbital. The ratio of RR/m values for diazepam to those of nordiazepam for (i.e., $(RR/m)_{DZP}/(RR/m)_{NDZP}$) each bone type are summarized in Figure 4. The mean $(RR/m)_{DZP}/(RR/m)_{NDZP}$ values ranged from 0.54 to 2.60.

Mass-normalized response ratios were analyzed by ANOVA (one-way) and Tukey's post-hoc analysis. For all three drugs, a significant ($p < 0.05$) main effect of bone type was observed.

Discussion

Data treatment

Analytical data from GC–MS assays are reported here in

terms of the mass-normalized response ratio (RR/m). This methodology is in keeping with standard quantitative methods used in GC–MS, in that the RR/m parameter is proportional to sample concentration and, therefore, serves as a good predictor of relative drug concentrations in different tissue types, but stops short of ascribing a concentration value to a given tissue sample. We prefer to report data in this manner because the determination of an absolute drug concentration in a bone sample is inherently complex as drug recovery from bone is often indeterminable and there are no standardized methods of sample preparation. Consequently, a reported drug concentration in a given bone sample may be expected to vary from laboratory to laboratory, and thus, concentration measurements may be misleading. Nonetheless, we have also reported the range of mean drug concentrations from the replicate extractions from each bone type. It must be underscored again that the utility of such concentration measurements is limited by the inability to estimate extraction recovery, and because such measurements, in the best case, only apply to these experimental conditions used here. Since the purpose of this work was to establish whether the different drugs examined were homogeneously distributed throughout the different skeletal tissues, the RR/m parameter was chosen as a suitable measure for comparison of analytical response to the different samples.

Statistical significance of results and interpretive implications

The RR/m data were examined by ANOVA (one-way) to determine whether the variability in RR/m values between different bone types was greater than that observed in multiple extractions of a given bone type. This type of analysis is important because bone tissue is solid and structurally heterogeneous, which implies that drug distribution may not be uniform within a given bone. ANOVA showed that the bone type sampled was a main effect with respect to measured RR/m values for each of the drugs analyzed. Examination of Figures 2 and 3 shows that for all drugs examined, the bone type with the largest RR/m measurement was rib. Large RR/m values were also found in vertebrae, femoral epiphyses, and pelvis. The range of variability with respect to tissue type was high, with the ratio of maximum to minimum RR/m values measuring 27, 39, and 20 for pentobarbital, diazepam, and amitriptyline, respectively.

The range in RR/m responses between different bone types suggest that such measurements may be limited in terms of providing a means to interpret toxicity. The general trend toward relatively larger responses in the bones in the vicinity of the central cavity was observed for all three drugs, and may be indicative of one of the mechanisms of drug deposition. As decomposition proceeds, the soft tissues liquefy, necessarily resulting in some liberation of drug partitioned within those tissues. Because bone is somewhat porous, the drug-rich fluid resulting from liquefying tissues may then be absorbed to some degree by the surrounding bone. The same phenomenon may apply to all bones, with the extent of absorption related to the amount of drug-laden tissue overlying the bone, the drug concentration in that tissue and the bone porosity. Further, the marrow type (yellow vs. red) varies based on anatomic site of the bone in question in humans (12). As red marrow is more highly vascularized than yellow marrow, there may be variations in drug distribution to the marrow throughout the body. Finally, different bone types may contain different relative amounts of dense cortical bone and spongy trabecular bone. Because the surface area exposed to marrow differs between the two forms, and diffusion of drug from marrow into solid bone may be expected to depend, at least partly, on this contact surface area, then site-dependent variation in bone drug levels seem reasonable. Overall, while the net effect may be a limitation in the ability to interpret drug measurements in skeletal tissues in terms of toxicity, this site dependence also implies that analysis of some tissue types may provide a greater likelihood of drug detection.

Given the significant variability in RR/m values observed between different bone types, one potential source of information about the drug exposure might be the relative amount of parent drug(s) and metabolite(s) in a given sample. The data in Figures 3 and 4 illustrate the relationship between measured RR/m values for diazepam and nordiazepam analyses in different bone types. ANOVA and post-hoc analysis showed that although bone type was a main effect for the observed RR/m in the case of diazepam (Figure 2), this was not the case for nordiazepam (Figure 3). Figure 4 illustrates the ratio of mean RR/m values for diazepam, relative to those of nordiazepam, $(RR/m)_{DZP}/(RR/m)_{NDZP}$, versus the mean RR/m values for diazepam. These data show substantial variability in the ratio of RR/m values for a particular tissue type but a positive correlation between RR/m ratios and the RR/m values for diazepam. ANOVA showed that bone type was a main effect with respect to $(RR/m)_{DZP}/(RR/m)_{NDZP}$, but the post-hoc analysis showed that the only significant difference between different bone types occurred between rib (maximum ratio of RR/m observed) and ulna (minimum ratio of RR/m observed). The data in Figure 3 show that the variability in RR/m values for nordiazepam was substantially larger than that observed in diazepam analyses. Overall, the ratio of mean RR/m values for diazepam relative to nordiazepam varied by a factor of 4.5. This may be influenced by differences in distribution of parent drug and metabolite to the tissues surrounding each bone type, and may also be influenced by the relative stability of the parent drug(s) and metabolite(s). Both of these effects may be at play in this particular case, given the observation of diazepam stability and nordiazepam instability in postmortem blood and tissues reported in the past (13). Overall, the investigative value of ratios of parent drug to metabolite will need to be the subject of future work, and variables such as drug and metabolite stability and pharmacokinetic phase should be controlled and their effects studied. An encouraging observation here was that the ratio of RR/m values for diazepam to those of nordiazepam showed less variability as a function of bone type than did the RR/m values themselves (4.5-fold variation for the RR/m ratio vs. 39-fold variation in RR/m for diazepam and 9-fold variation in RR/m for nordiazepam).

Conclusions

In this work, it was demonstrated that acute drug exposure could be detected through analysis of skeletal tissues of a porcine model after an extended (approximately two years) period of decomposition, for a number of different drugs with different chemical properties. Further, there appeared to be significant site-dependent distribution of these drugs between different skeletal tissues, ranging from as much as approximately 20-fold to 40-fold. Consequently, pending further research characterizing the magnitude of site-dependent variation in skeletal tissue drug levels, and standardization of methods for toxicological analysis of bone tissues, reporting of quantitative measurements of bone drug concentrations should be avoided.

Acknowledgments

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