Effects of Tissue Type and the Dose-Death Interval on the Detection of Acute Ketamine Exposure in Bone and Marrow with Solid-Phase Extraction and ELISA with Liquid Chromatography–Tandem Mass Spectrometry Confirmation

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Abstract

Ketamine exposure was detected in skeletal tissues by ELISA and liquid chromatography–tandem mass spectrometry (LC–MS–MS). Rats (n = 9) received ketamine hydrochloride acutely (75 mg/kg, i.p.) and were euthanized within 15, 30, or 90 min. Drug-free control animals (n = 3) were also euthanized. Extracted femora were separated into epiphyseal and diaphyseal fragments, with marrow isolated from the medullary cavity. Bone was ground and incubated in methanol. Extracts were dried and reconstituted in phosphate buffer (0.1 M, pH 7.3), and marrow was homogenized in alkaline solution. Both then underwent solid-phase extraction. Extracts were assayed by ELISA, with data expressed in terms of relative decrease in absorbance (%DA, drug-positive tissues vs. matrix-matched drug-free controls) and binary classification test sensitivity (S). Generally, %DA decreased in the order of marrow > epiphyseal bone > diaphyseal bone, and was negatively correlated with dose-death interval (DDI). Measured S values were 100% in ELISA analysis of extracts of all tissue types. Sensitivity values were computed from LC–MS–MS data using a 5 ng/mL cutoff. Sensitivity values for ketamine detection were 100%, 0–100% and 0%, at the 15, 30, and 90 min DDI, respectively, and sensitivity values for norketamine detection were 0–66%, 0–66%, and 0% at the 15, 30, and 90 min DDI, respectively. These results suggest that the tissue type sampled and DDI may influence the sensitivity of detection of ketamine exposure in skeletal tissues.

Introduction

Despite a number of recent reports in the literature addressing drug detection in skeletal tissues (mineralized bone and marrow), drug disposition in these tissues remains poorly understood (1–6). There are additional practical concerns in conducting further research into this area, because forensic analysis of human tissues requires the use of autopsy samples that are often poorly characterized in terms of drug use history, dose, and the like. Furthermore, the use of autopsy tissues in research may be problematic in many jurisdictions because of legal restrictions. As an alternative, animal models confer the required control over drug exposure, dose, and delay between drug administration and death (i.e., the dose-death interval). Animal models are advantageous from the perspective of understanding basic drug properties in tissues, such as distribution between tissue types, accumulation, and analytical method development. Such work may provide the necessary scientific basis to justify further research with human tissues so skeletal drug analysis may become validated for forensic field work. A variety of drugs have been measured in skeletal tissues using animal models, including members of the amphetamines (7), opiates (1,6), benzodiazepines (3–5), barbiturates (8,9), alcohols (10), and tricyclic antidepressants classes (11). However, extrapolation of data from animal models to human cases is not appropriate, especially in a forensic context.

As a result of the lack of basic research describing drug disposition in skeletal tissues, the determination of drugs of toxicological relevance in bone or marrow in forensic casework may be best reported in qualitative terms (e.g., through use of terms such as “detected”), as opposed to reporting of quantitative measurements. Consequently, drug detection in skeletal tissues may be interpreted as indicating exposure only because the ability to predict toxicity generally requires correlation of quantitative measurements with toxic effect. However, research involving qualitative or semi-quantitative measurements may still be useful in answering questions of forensic interest. For example, an understanding of how long a drug and/or its metabolites may be detected following a specific pattern of administration (e.g., acute vs. chronic) provides insight into the time window within which drug administration could have occurred.

In recent work (12), the utility of ELISA and gas chromatography with electron capture detection, in combination...
with a liquid–liquid extraction procedure, in the detection of ketamine exposure in assays of marrow and mineralized bone from rats acutely exposed to the drug was illustrated. The sensitivity of the ELISA response was shown to be dose-dependent, and it varied depending on the type of tissue examined (marrow, trabecular/epiphyseal bone, or cortical/diaphyseal bone). Here, an extension of this analytical approach is reported, with the detection of ketamine exposure via assay of extracts of skeletal tissues using automated solid-phase extraction (SPE) in combination with ELISA and qualitative confirmation by liquid chromatography–tandem mass spectrometry (LC–MS–MS). The purposes of this work were to examine the effect of the dose-death interval on both the assay response and the relative sensitivity of detection between tissue types and to further illustrate the applicability of techniques routinely used in the forensic toxicology laboratory to drug detection in skeletal tissues.

Experimental

Chemicals

Methanol used in drug extraction was high-performance liquid chromatography (HPLC) grade and obtained from EMD Chemicals (Gibbstown, NJ). Ketamine 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.

Animals and drug administration

The animals used were housed at the Laurentian University Animal Care Facility. Upon arrival at the facility, the animals were given seven days to acclimate to their conditions. Adult male Wistar rats (Charles River Laboratories, Saint-Constant, QC, Canada) were housed in groups of 2 with Harlan Teklad ¼ in. bedding (Indianapolis, IN) on a 12-h light/dark cycle at a room temperature of approximately 20°C. They were supplied with free choice water and Harlan Teklad Laboratory Diet 8640. Twelve adult male Wistar rats were given 0 (n = 3) or 75 mg/kg (n = 9) ketamine hydrochloride (i.p.). Drug-positive rats were euthanized within 15, 30, or 90 min with CO2 gas, and control animals were euthanized at the onset of the study period. These dose-death intervals were chosen based on an estimated half-life of ketamine of approximately 37 min in rats (13). Hindlegs were removed and separated into diaphyseal and epiphyseal sections using a rotary tool with a cutting wheel. The marrow was extracted from the medullary cavity by syringe. Marrow from the left and right femora were extracted separately and then subsequently pooled to prevent saturation of the SPE resin, and bone tissues from the left and right femora were pooled according to tissue type (diaphyseal and epiphyseal) prior to analysis, to maximize sensitivity.

Marrow preparation

Isolated marrow was weighed and dissolved in 3 mL 0.25 M NaOH/0.25 M NaCl with ultrasonication. Marrow homogenates were diluted 2:1 with phosphate buffer (0.1 M NaH2PO4, pH 6) to yield a final pH of approximately 7. The resultant solutions then underwent SPE as described.

Bone preparation

After tissues adhering to the surface of the bones had been removed by scraping with a scalpel, epiphyseal portions of each femur were separated from the diaphyses. Each portion was immersed in 5 mL 0.25 M NaOH/0.25 M NaCl overnight, and subsequently cleaned by ultrasonication for 30 min. The medullary cavities were then flushed with this washing solution, and the samples then underwent further ultrasonication for 30 min. The wash solution was discarded and the bones were subsequently rinsed with deionized water (dH2O) and methanol, and then dried under a steady flow of argon at 50°C. The bones were then ground in a general purpose domestic grinder, followed by further grinding in a mortar and pestle.

Dried bone was accurately weighed into 20-mL threaded glass test tubes. Methanol (3 mL) was then added to each tube. The samples were incubated at 50°C for 72 h. Following isolation and centrifugation (3000 rpm, 10 min), supernatants were transferred into clean glass test tubes and evaporated under argon at 50°C. Residues were then reconstituted in 0.1 M phosphate buffer (pH 7.3, 3 mL) and underwent SPE.

SPE

Marrow homogenates and reconstituted bone extracts underwent SPE using Bond-Elut Certify columns (3 mL/50 mg, Varian, Mississauga, ON, Canada) using a Gilson ASPEC XL-274 Automated SPE instrument (Middleton, WI). Columns were conditioned with 2 mL methanol, 2 mL dH2O, and 1 mL pH 4.5 acetate buffer. Samples were loaded (2 mL) at a flow rate of 0.1 mL/min, and columns were sequentially washed with acetic acid buffer (pH 4.5, 2 mL), 1 M acetic acid, and 2% acetic acid in 25:75 methanol/dH2O (2 mL). Elution was accomplished with 5% NH4OH in methanol (2 mL). All eluted fractions were evaporated to dryness under argon (50°C) and subsequently reconstituted in dH2O (1 mL).

ELISA

Immuoassay of ketamine in extracts of marrow and mineralized bone were made using commercially available ELISA kits (Immunalysis, Pomona, CA), as per the manufacturers instructions. Immunoassay was automated using a ChemWell® 2910 Automated EIA and Chemistry Analyzer (Awareness Technologies, Palm City, FL). The analytical protocol used a microwell plate temperature of 25°C. In each microwell, 10 µL of the aqueous sample solution was combined with 100 µL of diluted enzyme conjugate. The plate was shaken gently for 10 s and allowed to incubate for 60 min without agitation. The wells were then washed 3 times with 100 µL of phosphate buffered saline solution (pH 6.5). Following aspiration of the wash solution, 150 µL of enzyme substrate (3,3′,5,5′-tetramethylbenzidine, TMB) was added to each well and allowed to incubate for 30 min at 25°C. The reaction was stopped by addition of 50 µL of 1 N HCl stop solution to each well, followed by gentle agitation for 10 s. The absorbance of each well was then measured at 450 nm.

LC–MS–MS

All samples underwent further analysis by LC–MS–MS.
Aqueous extracts were evaporated to dryness by vacuum centrifugation following analysis by ELISA, and reconstituted in 50 µL containing 500 ng/mL internal standard (ketamine-d₄ and norketamine-d₄). An Agilent Technologies 1200 series LC pump coupled to a 6410 triple-quadrupole MS operating in positive atmospheric pressure chemical ionization mode was used for analysis. The LC column was a Zorbax Eclipse XDB C18 (4.6 × 50 mm × 1.8 mm) held at 40°C, and the injection volume was 2 µL. The mobile phase consisted of 20 mM ammonium acetate (Solvent A, pH 6.4) and methanol (Solvent B), and both the composition of the mobile phase and the flow rate were varied throughout each analysis. The system was allowed to re-equilibrate for 7 min between analyses. The gas temperature was 350°C, the gas flow was 5 L/min, and the nebulizer pressure was 50 psi. Nitrogen was used as the collision gas, and the capillary voltage was 4500 V. The flow timetable as well as optimized ion transitions, fragment voltages and collision energies are shown in Tables I and II. Under these conditions, the retention times (tᵢ) for ketamine and norketamine were 6.12 and 6.09 min, respectively. Ketamine and norketamine were considered to be identified when measured tᵢ values were within 5% of the corresponding standard and within 0.1 min of the corresponding deuterated internal standard. Further, the ratio between the monitored transitions had to be within 20% of the ratio observed in calibration standards in order to be considered positive. The cutoff concentration used in the LC–MS–MS assay was 5 ng/mL for both ketamine and norketamine.

Results

Performance characteristics of the ELISA method for ketamine detection—precision, concentration dependence of response, and cross-reactivity

For purposes of data analysis, raw absorbance data were analyzed directly and transformed to determine the relative decrease in absorbance, expressed as a percentage (%DA) of the mean absorbance of the set of drug-free control samples, according to equation (1):

\[
% \text{ Decrease in Absorbance} = 100% \times \frac{(A_{\text{ctrl}} - A)}{A_{\text{ctrl}}} \quad \text{Eq. 1}
\]

where A is the mean absorbance of a given sample and A_{\text{ctrl}} is the mean absorbance value of a set of matrix-matched, drug-free control tissue extracts. The precision of replicate analyses (i.e., coefficient of variation with respect to raw absorbance measurements generated from duplicate assays of a given sample extract) of a given bone extract ranged from 0.0 to 5.0%. The precision of replicate analyses of a given marrow sample ranged from 0.1 to 7.8%. In this work, equal volumes of reconstituted marrow extracts from right and left limbs were combined in order to generate an assay response corresponding to the average of the two marrow samples, because substantial variability between marrow samples from different limbs was observed, with coefficients of variation as large as 44% observed in earlier work (12). This variability is likely due to the combined effects of biological variability in terms of drug distribution into the viscous marrow matrix, differences in mass of marrow sampled between different limbs, and the variability associated with measurements made in different microwells.

Because of the non-linearity of the ELISA response with respect to ketamine concentration, extraction efficiency cannot be accurately determined using this method. However, comparison of absorbance values of extracted and unextracted standards at various concentration levels over the range of 5–200 ng/mL showed coefficients of variation ranging from 4.1 to 30.6%. Both imperfect extraction efficiency and imprecision of replicate measurements between microwells contribute to this observed variability.

Concentration dependence of ELISA response

Matrix-matched standard solutions of ketamine were prepared (0–200 ng/mL) and extracted by the SPE protocol described. Extracts were then assayed by ELISA. Figure 1 illustrates the concentration-dependent %DA measurements as a function of ketamine concentration in spiked bone samples using this assay.

ELISA cross-reactivity studies—endogenous compounds within the skeletal tissue matrix

As was done in earlier work (12), a series of control tissue samples derived from drugfree animals underwent the extraction and analysis procedures along with those derived from drug-positive animals. In this work, comparison of assay response to drug-free extracts from epiphyseal and diaphyseal bone showed significant differences in response between the two groups (p < 0.05). This may have been due to differences in mass of the pooled epiphyseal versus pooled diaphyseal bone fragments, and underscores the need for using properly matrix-matched control samples in studies such as this. The cross-reactivity of the Immunalysis ELISA kits was also evalu-

<table>
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<tr>
<th>Drug</th>
<th>Precursor Ion</th>
<th>Fragment Ion</th>
<th>Fragmentor Voltage (V)</th>
<th>Collision Energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketamine-d₄</td>
<td>242.2</td>
<td>129</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>Ketamine</td>
<td>238.1</td>
<td>179.2</td>
<td>120</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>238.1</td>
<td>125</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Norketamine-d₄</td>
<td>228.1</td>
<td>129</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>Norketamine</td>
<td>224.1</td>
<td>207.1</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>224.1</td>
<td>125.2</td>
<td>80</td>
<td>25</td>
</tr>
</tbody>
</table>

Table II. Ion Transitions and Associated Fragmentation Voltages and Collision Energies used in LC–MS–MS Analysis
uated with respect to varying concentrations of norketamine. In these experiments, norketamine solutions (5–500 ng/mL) were assayed and generated %DA values ranging from 6.6 to 68.6%, corresponding to a cross-reactivity of approximately 5% at a norketamine concentration of 100 ng/mL.

**Relative sensitivity of detection: effects of tissue type and dose-death interval**

Relative decreases in absorbance for tissue extracts (%DA) were computed for each experimental condition (tissue type, dose-death interval). Figure 2 presents a plot of the mean uncorrected absorbance values (A) and the corresponding mean %DA values measured in each experimental condition (B). Relative decreases in absorbance observed in assays of extracts of marrow were significantly greater than those of extracts of epiphyseal bone ($p < 0.05$) at all dose-death intervals examined. Similarly, %DA values observed in extracts of epiphyseal bone were significantly greater than those of diaphyseal bone ($p < 0.05$) at all dose-death intervals examined.

The effect of the dose-death interval on the observed %DA values was also examined. For marrow extracts, the %DA values at the 90 min dose-death interval were significantly different than those at both the 15 and 30 min dose-death intervals, respectively ($p < 0.05$), but the %DA values at the 15 and 30 min dose-death intervals did not differ significantly. For epiphyseal extracts, the %DA values at the 90 min dose-death interval were significantly different than those at both the 15 min and 30 min dose-death interval ($p < 0.05$), but no significant differences were observed between the 15 and 30 min dose-death intervals. For diaphyseal extracts, there were no significant differences between %DA values over any of the three dose-death intervals examined. Mean %DA values were negatively correlated with dose-death interval for all values examined. Correlation coefficients ($r$) were $-0.83$, $-0.85$, and $-0.70$ for extracts of marrow, epiphyseal bone, and diaphyseal bone, respectively.

**Binary test sensitivity of ketamine detection in marrow, epiphyseal bone, and diaphyseal bone**

The use of ELISA as a screen for ketamine exposure in a given skeletal tissue extract represents a binary classification test, where ketamine exposure may be considered to be detected if the ELISA response varies significantly relative to those of an appropriate population of matrix-matched control samples. As such, the sensitivity of the ELISA analysis for ketamine in bone tissue may be expressed as

$$\text{Sensitivity (S)} = 100\% \times \frac{TP}{FN+TP}$$

where $TP$ represents the number of true positive detections (i.e., cases where the ELISA response corresponded to detection of ketamine in drug-positive tissues) and $FN$ represents the number of false negative detections (i.e., cases where the ELISA response to extracts of drug positive tissues did not differ significantly from those of drug-free controls). This methodology has been applied to the evaluation of immunoassay performance in a number of studies by Spiehler et al. (14,15). In this work, ketamine was considered to be detected in a given sample if the ELISA response (i.e., absorbance at 450 nm) was less than the mean absorbance of drug free extracts of the corresponding tissue, minus three standard deviations. The absorbance value from each sample assayed was compared against the mean absorbance value from the appropriate control group (i.e., marrow, epiphyseal bone or diaphyseal bone) and a designation of detected or not detected was assigned based on this definition. As such, the binary classification sensitivity of the ELISA
assay was determined for all tissue types at all dose-death intervals (Table III).

**LC–MS–MS analysis of skeletal tissue extracts**

Using a 5 ng/mL cutoff for the LC–MS–MS method, ketamine was detected in all of the marrow samples examined over all of the dose-death intervals investigated. Ketamine was detected in 100% of the epiphyseal bone extracts corresponding to the 15 and 30 min dose-death intervals, but in none of the epiphyseal bone extracts corresponding to the 90 min dose-death interval. Ketamine was not detected in any of the diaphyseal bone extracts examined. Norketamine was detected in 33% of the marrow extracts at the 15 min dose-death interval, 100% of the marrow and 33% epiphyseal bone extracts corresponding to the 30 min dose-death intervals, but in none of the marrow or epiphyseal bone extracts corresponding to the 90 min dose-death interval. Norketamine was not detected in any of the diaphyseal bone extracts examined. These data are summarized in Table III. Examples of LC–MS–MS chromatograms are shown in Figures 3 and 4.

**Discussion**

**Value and limitations of experimental approach**

The purpose of this work was the examination of the utility of ELISA for the examination of the effects of the dose-death interval on the detection of ketamine exposure in skeletal tissues. This involved the use of an animal model under controlled experimental conditions of drug administration. In contrast to the examination of autopsy tissues, the use of experimental animals is a significant departure from the conditions encountered in forensic casework. However, it does represent a means to control forensically relevant factors such as dose, route of administration, drug use history, and dose-death interval. This approach allows for the direct comparison of the assay response in skeletal tissues that are known to be drug-free with those from animals known to have been exposed to the drug of interest. In comparing assay responses within this study, various experimental variables were controlled for, including tissue type and the drug exposure history of the animals.

ELISA was chosen to compare relative sensitivity of detection of ketamine exposure in skeletal tissue samples for a number of reasons. The assay has been shown to display a concentration-dependent response and sufficient sensitivity to detect ketamine at concentrations of approximately 5 ng/mL, or perhaps lower (Figure 1). Although ELISA is typically used in the field as a qualitative screening tool to presumptively indicate the presence of a drug (or member of a family of drugs) above a threshold level, the semiquantitative nature of the assay response implies that it may be able to provide important information as to the best sites for bone tissue sampling and the effects of dose-death interval on assay response. In addition, other variables affecting the likelihood of drug detection that may arise in a forensic investigation can be explored, including the pattern of drug use and postmortem environment. This will be valuable in understanding the implications and limitations of drug detection in skeletal tissues, even if the results are considered only in qualitative terms. Finally, ELISA is a technique that is widely used across many forensic laboratories, although its utility in this application has gone largely unexplored.

Because ELISA, like other immunoassays, is susceptible to signal generation from any compounds that may bind to the immobilized antibodies (i.e., cross-reactivity), the response of the assay to co-extracted compounds endogenous to the skeletal tissues was characterized. The contributions of the ketamine metabolite norketamine and endogenous compounds were shown to be relatively low. However, there may still be additional contributions to the observed assay response from other cross-reacting compounds. This was controlled for, to some extent, through the use of matrix matched control samples and the use of the %DA parameter, which expresses the assay response to tissue extracts from drug positive animals relative to that from drug-free extracts of the same tissue type. Furthermore, positive ELISA results are discussed in the context of being indicative of ketamine exposure, which takes into consideration the potential contribution from ketamine metabolites. The addition of a sensitive LC–MS–MS assay

<table>
<thead>
<tr>
<th>Dose-Death Interval (min)</th>
<th>Tissue Sampled</th>
<th>Mean % DA ± S.D. (Range)</th>
<th>Sensitivity (%) (ELISA)</th>
<th>% Samples with Ketamine Detected (LC–MS–MS)</th>
<th>% Samples with Norketamine Detected (LC–MS–MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 Marrow</td>
<td></td>
<td>79.1 ± 3.4 (75.3–81.9)</td>
<td>100</td>
<td>100</td>
<td>33</td>
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<tr>
<td>15 Epiphyseal bone</td>
<td></td>
<td>66.8 ± 5.0 (63.0–72.4)</td>
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<td>0</td>
</tr>
<tr>
<td>15 Diaphyseal bone</td>
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<td>38.3 ± 2.1 (36.8–40.7)</td>
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<td>30 Marrow</td>
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<td>79.2 ± 4.8 (73.8–82.9)</td>
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<tr>
<td>30 Epiphyseal bone</td>
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<td>57.8 ± 11.8 (49.5–66.2)</td>
<td>100</td>
<td>100</td>
<td>33</td>
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<tr>
<td>30 Diaphyseal bone</td>
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<td>25.7 ± 2.7 (22.7–26.8)</td>
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<tr>
<td>90 Marrow</td>
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<td>69.7 ± 1.4 (68.0–70.6)</td>
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<tr>
<td>90 Epiphyseal bone</td>
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<td>31.9 ± 16.9 (12.9–45.2)</td>
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<td>0</td>
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<tr>
<td>90 Diaphyseal bone</td>
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<td>20.9 ± 9.7 (15.2–32.1)</td>
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</tr>
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</table>
capable of resolution and identification of ketamine and norketamine was valuable in confirming the presence of ketamine, and in indicating the likelihood of a significant contribution to the ELISA response from norketamine.

The utility of the binary classification sensitivity parameter as a measure of relative sensitivity of detection of ketamine exposure depends on the limit of detection of the analytical method used, and the sample preparation steps undertaken. In this work, values of $S$ for the ELISA assay were 100% for all tissue types and dose-death intervals examined, whereas the data in Table III show that the frequency of detection of ketamine and norketamine by LC–MS–MS was substantially lower than the frequency of positive ELISA results for some tissue types and dose-death intervals. Although the binary classification sensitivity data obtained using ELISA may appear to suggest that detection of ketamine exposure is equally probable for all of the tissues investigated at all dose-death intervals, there are limitations in applying this parameter as the only criteria in evaluating the relative sensitivity of detection of drug exposure via immunoassay. Importantly, the %DA measurements, along with confirmatory LC–MS–MS measurements are valuable in indicating that sensitivity of detection of ketamine exposure decreases as the dose-death interval increases. Measured %DA values facilitate direct comparison of semi-quantitative responses. Given that the %DA is concentration dependent and the data in Figure 2 show that the %DA decreased as the dose-death interval increased, then the data suggests the dose-death interval may have a significant effect on the sensitivity of ELISA response. The dose-death intervals examined here represent those that are less than that typically required for complete drug clearance from the blood (i.e., $5t_{1/2}$), which suggests that significant reductions in detection sensitivity occur within relatively short-dose death intervals following acute administration. If this is replicated in human tissues, and the trend extended to longer dose-death intervals,
then positive drug screen results, pending confirmation, may be indicative of recent ketamine exposure. Further work examining the time course of ketamine detection, including the effects of extended dose-death intervals will be forthcoming.

The differences in measured sensitivity values between ELISA and LC–MS–MS analyses may seem to suggest ELISA measurements of diaphyseal bone extracts and those of epi-
physyal bone extracts at the 90 min dose-death interval repres-
sent false-positive results because the presence of ketamine and/or norketamine was not confirmed by LC–MS–MS. Although this is an appropriate conclusion in forensic casework, it must be noted in this case that the analytical sensitivity of the ELISA method is comparable to that of the LC–MS–MS assay, which has an associated cut-off concentration of 5 ng/mL, and the use of the relative decrease in absorbance helps protect against false-positive signal generation due to endoge-
nous matrix components. Examination of the data in Figure 1 show that 5 ng/mL solutions were clearly detectable by ELISA, and consideration of signal-to-noise ratios suggest that significant %DA values may in fact be observed at even lower con-
centrations. Ultimately though, ELISA remains incapable of definitive identification of the presence of a compound in a given sample. Consequently, there is some uncertainty regarding the true reason for the discrepancy between the ELISA and LC–MS–MS data, so calculation of specificity values (related to the probability of false negative results) is not appro-
priate at this point. This illustrates the need for ongoing research and development of definitive analytical methods with very high sensitivity for the identification of ketamine and other drugs in skeletal tissue matrices.

Implications of detection of ketamine in marrow, epi-
physyal bone, and diaphyseal bone

In aggregate, these results agree with earlier work (12), sug-
gesting that acute ketamine exposure can be reproducibly de-
tected by ELISA in marrow and mineralized epiphyseal bone, while measurements using diaphyseal portions were less reli-
able. The data in Figure 2 and Table III show that the sensitivity of ketamine detection is greatest for marrow, and is greater for epi-
physyal bone in comparison to diaphyseal bone, when esti-

ted via both the relative decrease in absorbance and the bi-
nary classification test sensitivity of the ELISA method. The reason for differences in sensitivity of response between dia-
physyal and epi-
physyal tissue remain unclear, but may be due to differences in drug distribution, or differences in extraction efficiency from these tissues.

In this work, bone fragments were pooled according to tissue type for each animal (i.e., left and right epiphyses were pooled) prior to drug extraction, as opposed to weighing a targeted mass of each type of bone for extraction after cleaning and pul-
verization. This methodology ensured that significant portions of drug positive bone were not excluded in sampling, and was done to improve the likelihood of drug detection at the longer dose-death intervals examined. However, there were signifi-
cant differences between the masses of the pooled diaphyseal fragments and the pooled epiphyseal fragments. Further, there were observable differences between the ELISA response to pure deionized water and that of drug-free tissue extracts, where mean ELISA absorbance values for drug-free deionized water, marrow extracts, epiphyseal bone extracts and diaphyseal bone extracts were 2.18 ± 0.10, 2.27 ± 0.06, 1.72 ± 0.05, and 1.98 ± 0.04, respectively. Thus, it is likely that there may be some endoge-
nous compounds co-extracted that provide some contribu-
tion to the ELISA response. When comparing assay responses within a given tissue type, this is mitigated to a large extent through the use of the %DA parameter. However, when compar-
ing drug positive tissues of different types with significant differences in mass, the effect of the mass of tissue on ELISA re-

sponse may be estimated through normalization of %DA with respect to mass (i.e., %DA/m), although these effects should be interpreted qualitatively only because of the non-linear relationship between ELISA response and analyte concentration. Comparison of normalized %DA values highlights the difference in detection sensitivity for ketamine exposure in different tis-

ues. Differences in normalized %DA values between marrow and epiphyseal bone extracts increase sharply because the mass of marrow assayed in this work was approximately 0.04 g, whereas that of epiphyseal bone was approximately 0.8 g. Con-
versely, differences in the normalized %DA values for epi-
physyal bone and diaphyseal bone were reduced relative to non-
nor-

malized values although the same general trend with respect to dose-death interval was observed in mean values (Figure 5). In the separation of epiphyseal and diaphyseal portions, there may be some cortical bone from the diaphyseal fragment that is un-

intentionally collected with the epiphyseal fraction. Further-
more, there are components of both trabecular and cortical bone in each fragment type; epiphyseal fragments are com-
posed primarily of trabecular bone, but contain some cortical bone as well, while the opposite is true in diaphyseal fragments. Overall, this is a potentially confounding influence in estab-

lishing relative sensitivity of response. However, in previous work (12), where fragments of a given tissue type recovered from left and right limbs were not pooled, there was no signifi-
cant difference between bone masses, and %DA values were sig-
nificantly greater in epiphyseal bone than in diaphyseal bone. A further point of interest arising from the normalized %DA data in Figure 5 is the plateau of the %DA/m data at non-zero values for diaphyseal bone extracts at the 30 min and 90 min dose-
death intervals. Given that these measurements are relative to matrix-matched, drug-free control tissues it seems somewhat unlikely that this is due to an endogenous matrix component.

Alternatively, if the measured response at this level is due to ex-
tracted drug, then this may be representative of drug that was somewhat “trapped” within the bone matrix, or at least quite slow in diffusing out of the tissue as metabolism proceeded. Again, clarification of this phenomenon will require the develop-

ment of a highly sensitive quantitative method for ketamine analysis with sufficient informing power as to reliably identify ketamine or ketamine metabolite in a given sample for further analysis of both blood and bone tissue extracts at extended dose-death intervals.

The data presented here may have significant implications for drug screening and analysis in human tissues. If the site-de-

pendent ELISA sensitivity observed here is reflective of het-

erogeneous drug distribution in a given bone, then the possi-

bility that partial bone sampling (as would likely be done
in analyses of human bone tissues) may yield no detectable drug concentrations even when drug exposure has occurred. Clarification of this issue will require the development of a significant body of data using human bone tissue to determine the appropriate site and number of samples to include from a given bone region to minimize the risk of false negatives.

Conclusions

These data have shown that ELISA may be a valuable tool in screening of skeletal tissue samples for drugs of abuse. Marrow appears to be a significant depot for ketamine distribution. These results support earlier work suggesting that epiphyseal bone fragments may provide a more reliable and sensitive sampling site than diaphyseal bone from the mid-femoral region for studies of ketamine disposition in skeletal tissues and suggest that the time delay between drug administration and death (the dose-death interval) may have a substantial effect on the sensitivity of drug detection in skeletal tissues. Significant reductions in detection sensitivity were observed over relatively short dose-death intervals, which may suggest that positive drug screen results in skeletal tissues may be indicative of recent exposure. More research is required involving the examination of the effects of longer dose-death intervals on the sensitivity of drug detection, including other drugs of forensic relevance with different chemical and pharmacological properties.

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