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Trevor C. VandenBoer,[1] *B.Sc. (Hons.); Samuel A. Grummett,*[1] *B.Sc. (Hons.); and James H. Watterson,*[1] *Ph.D.*

Utility of Immunoassay in Drug Screening in Skeletal Tissues: Sampling Considerations in Detection of Ketamine Exposure in Femoral Bone and Bone Marrow Following Acute Administration Using ELISA*

ABSTRACT: Detection of ketamine exposure in skeletal tissues by automated enzyme-linked immunosorbent assay (ELISA) and gas chromatography with electron capture detection (GC-ECD) is described. Rats (n = 18) received 0, 15, 30, or 75 mg/kg ketamine hydrochloride acutely (i.p.), and were euthanized within 15 min or 1 h. Ketamine was extracted from ground femoral bone by methanolic incubation followed by liquid–liquid extraction (LLE), while marrow was homogenized in alkaline solution, and then underwent LLE. Extracts were analyzed by ELISA, and subsequently by GC-ECD following derivatization with trifluoroacetic acid anhydride. The effect of tissue type (i.e., diaphyseal bone vs. epiphyseal bone vs. bone marrow) on the immunoassay response was examined through determination of binary classification test sensitivity (S) and measurement of the relative decrease in absorbance (%DA, drug-positive tissues vs. drug-free controls) in each tissue type. The %DA varied significantly between different tissues examined under a given dose condition, and generally decreased in the order marrow > epiphyseal bone > diaphyseal bone, at all dose levels examined. Measured S values for marrow, epiphyseal bone, and diaphyseal bone were 100%, 77%, and 23%, respectively (75 mg/kg dose). These results suggest that the type of skeletal tissue sampled and position sampled within a given bone (diaphyses vs. epiphyses) are important parameters in drug screening of skeletal tissues.

KEYWORDS: forensic science, toxicology, bone, marrow, drugs, ketamine, immunoassay, gas chromatography

In death investigations involving skeletal remains, the availability of sample matrices conventionally used for toxicological analysis, such as blood, urine, visceral tissues, or even hair, may be significantly limited. In some cases, skeletal tissues may be the only source of toxicological information. However, interpreting the implications and limitations of toxicological analyses in skeletal tissues requires an understanding of drug disposition in bone and bone marrow, including the spatial distribution and time course of drug concentration profiles in those tissues, which may vary as a function of the chemical and pharmacological properties of the drug in question.

Currently, the scientific literature is limited in reports of controlled investigations of drug disposition in skeletal tissues. While some case reports have been published describing detection of various drugs in human bone and bone marrow (1–8), these reports are limited by a lack of information regarding the history of drug use in the decedent, including information regarding drug tolerance, dose, and time between drug administration and death. As the availability of such data in studies of human tissues is often significantly limited, analytical method development and drug disposition studies are often done using animal models, where conditions, such as drug use history, dose, time between drug administration and tissue collection and postmortem environment, can be more rigorously controlled. A number of such studies have been done, with various drugs measured in mineralized bone (9,10) and bone marrow (10–14). However, quantitative measurements in such studies have limited value in terms of direct applicability to human subjects.

Furthermore, there have been few reports describing the spatial distribution of drugs within a given bone or between different bones to determine whether specific bones or bone fragments are more suitable for drug screening than others. The value of such information is underscored by the structural heterogeneity that exists within and between the various bones in the skeleton, and by the potential for recovering only skeletal fragments in forensic casework. When remains are sufficiently unprotected to allow scavenging to occur, bones may be scattered, and those recovered may have been broken by animals seeking the encased marrow as a food source. A number of reports of drug detection in human bone have included tissues isolated from the femur (5,6,8), while others have reported analysis of vertebra (1) and rib (3). Those studies reporting analysis of drug concentrations in femoral bone have relied on sampling bone segments from the mid-section of the bone, with no attention paid to the trabecular bone within the epiphyseal regions. Most controlled animal studies published to date have reported drug concentrations in bone marrow, with only very few examining drug concentration in mineralized bone. Overall, while a growing body of literature is illustrating that drug detection in skeletal tissues is possible, important work remains to develop an understanding of the distribution characteristics of different drugs between different bones and within a given bone

[1]Forensic Toxicology Research Laboratory, Department of Forensic Science, Laurentian University 935 Ramsey Lake Rd. Sudbury, ON, Canada P3E 2C6.

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so as to understand the utility of drug concentration measurements in skeletal tissues.

Studies characterizing skeletal drug disposition using animal models may require the analysis of a large number of samples when it is considered that for each single experimental condition of dose, pattern of administration (i.e., acute vs. chronic administration), time delay between administration and tissue collection and postmortem environment, a number of tissue samples must be collected, prepared, and analyzed. Efficient and total separation of marrow from mineralized bone is required, along with the collection of multiple bones from each animal in the set of replicates. For each condition, it is reasonable to expect five to ten samples requiring analysis for each animal, creating a scenario where hundreds of samples may require analysis. In such scenarios, the use of immunoassay screening methods is valuable in improving analytical efficiency through reducing the number of samples that undergo more time consuming analyses by chromatographic methods. Immunoassay methods have the advantage of high sensitivity and the potential for high throughput facilitated by the parallel nature of analysis in a microwell format. Additionally, immunoassay is pervasive in clinical and forensic drug testing laboratories as these methods have proven reliable in the presumptive indication of the presence of a drug in a biological sample at a concentration above a cutoff value. While they may be limited in terms of selectivity as a result of potential cross-reactivity between antibodies and drug metabolites or endogenous matrix components, the appropriate use of control samples, sample preparation, and validation of response may minimize the incidence of false positive results.

A variety of drugs have been determined in skeletal tissues, including members of the opiates (e.g., morphine, 6-acetylmorphine) (5,10), benzodiazepines (e.g., flurazepam, midazolam) (9,13), and tricyclic antidepressants (11). Ketamine is a valuable candidate to add to this catalog of drugs that may be detected in skeletal tissues. Ketamine is a dissociative anesthetic with fewer adverse side effects compared with the structurally and pharmacologically similar compound phencyclidine (15). Ketamine is used recreationally (16) and has been implicated in cases of drug-facilitated sexual assault (17). Ketamine hydrochloride is a racemic mixture manufactured as a white crystalline powder and is dissolved in water for medical use in anesthesia for short diagnostic and surgical procedures. Ketamine is a weakly basic compound ($pK_a = 7.5$) with a moderate volume of distribution ($V_d = 3–5$ L/kg) and it has been shown to significantly partition into the brain and liver of humans (18). In humans, ketamine is rapidly distributed, and has a relatively short elimination half-life of 2–4 h (19). The metabolism of ketamine to its metabolites norketamine and dehydronorketamine occurs primarily in the liver and all three compounds are then largely hydroxylated and eliminated from the body by the kidneys as conjugates (20). The pharmacological characteristics of ketamine in rats have also been investigated. Williams et al. (21) reported a mean volume of distribution of 6 L/kg and elimination half-life ranging from 20 to 43 min; anesthetic doses of 101 ± 3 mg/kg (21,22) have been reported. These pharmacological data differ somewhat from those reported for humans, highlighting the need to remain cognizant of the potential differences in pharmacokinetic profile for a given drug between different species when conducting studies with animal models.

To date, detection of ketamine in skeletal tissues has not been described in the literature. Given the forensic relevance of ketamine, the commercial availability of immunoassay kits for screening ketamine in biological samples, and the pervasiveness of immunoassay in forensic drug screening applications, the purpose of this work was to determine whether immunoassay in the enzyme-linked immunosorbent assay (ELISA) format was suitable for the detection of ketamine exposure in marrow and mineralized bone of rats acutely exposed to ketamine hydrochloride. As it may be an important sampling consideration in forensic casework, the effect of the tissue type (i.e., marrow, cortical bone, or trabecular bone) on the sensitivity of the ELISA response was examined in two different ways. First, the semi-quantitative nature of the ELISA assay was exploited to compare the relative change in ELISA response (i.e., absorbance) in each tissue type under a given dose condition, using matrix-matched extracts derived from drug-free animals as reference values. As ELISA can be shown to provide a concentration-dependent change in absorbance, consideration of the relative differences in ELISA response of tissue extract derived from drug-free and drug-positive animals provides a measure of the relative sensitivity of detection of ketamine exposure between different tissue types for method development purposes. Second, raw ELISA absorbance data were analyzed in terms of binary classification sensitivity, which is an expression of the probability of a positive test result in the assay of tissues known to have come from animals exposed to ketamine. This approach also provides information on the reliability of a given tissue type for detection of ketamine exposure under a given set of experimental conditions.

All tissue extracts were also assayed by a qualitative analysis based on gas chromatography with electron capture detection (GC-ECD) following derivatization with trifluoracetic acid anhydride (TFAA), to provide supportive information on the presence of ketamine and the primary metabolite, norketamine, based on retention time data. Overall, this work was intended to examine the viability of ELISA as both a forensic screening tool in skeletal tissues, and as a source of information on the relative likelihood of detection of ketamine exposure in different skeletal tissues (i.e., bone vs. marrow) as well within different locations from a given type of bone.

Experimental Methods

Chemicals

Methanol and toluene used in drug extraction were HPLC grade and were obtained from EMD Chemicals (Gibbstown, NJ). Ketamine and norketamine standards (Cerilliant, Round Rock, TX) were obtained as 1 mg/mL methanolic solutions and diluted as required. TFAA (99%) was obtained from Sigma Aldrich (Oakville, ON). All other chemicals were reagent grade and were obtained from EMD Chemicals.

Animals & Drug Administration

The animals used were housed at the Laurentian University Animal Care Facility. Upon arrival at the facility, the animals were given 7 days to acclimatize to their conditions. Adult male Wistar rats (Charles River Laboratories, Saint-Constant, QC) were housed into two groups with Harlan Teklad 1/4″ bedding (Indianapolis, IN) on a 12 h light/dark cycle at a room temperature of *c.* 20°C. They were supplied with free choice water and Harlan Teklad Laboratory Diet 8640.

Animals were treated in two separate groups. One group of animals (group 1) was used to examine the effect of varying ketamine dose on measured bone tissue concentrations. This study involved eight adult male Wistar rats, given 0, 15, 30, 75 mg/kg ketamine (i.p., $n = 2$ per dose) and euthanized within 1 h with CO_2 gas. Further investigation of spatial distribution and analytical variability was done with tissues from another group of animals (group 2)

donated by Dr. T.C. Tai (Northern Ontario School of Medicine) that were used as part of a separate, unrelated research project. This group consisted of 10 adult male Wistar rats. Drug-positive rats received 75 mg/kg ketamine and 5 mg/kg xylazine (i.p., $n = 7$) and were subsequently killed within 15 min with CO_2 gas, with the remaining three animals being used as drug-free controls. The remains were frozen (−20°C) until processing, where left and right femoral bones were removed and separated into diaphyseal and epiphyseal sections. The marrow was extracted from the medullary cavity by syringe. Bones from the left and right legs were analyzed separately.

Marrow Preparation

Isolated marrow was weighed and subsequently dissolved in 3 mL of a solution containing 0.25 M NaOH/0.25 M NaCl with ultrasonication. The resultant solutions then underwent liquid–liquid extraction (LLE) as described below.

Bone Preparation

After tissues adhering to the surface of the bones had been removed by scraping with a scalpel, epiphyseal portions were separated from the diaphyses. Each bone fragment was placed in a separate 20 mL threaded glass test tube and immersed in a 5 mL 1:1 (v/v) solution of 0.5 M NaOH and 0.5 M NaCl overnight, and subsequently cleaned by ultrasonciation for 30 min. The medullary cavities were then flushed with washing solution and the samples were then ultrasonicated for an additional 30 min. The wash solution was discarded and the bones were rinsed briefly with methanol and dried under a steady flow of inert gas 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 (*c*. 0.3 g) was accurately weighed into 20 mL threaded glass test tubes. Methanol (3 mL) was then added to each tube. Negative control samples included both bone from drug-free animals as well as tubes containing only solvent (i.e., no bone added). The samples were incubated at 50°C for 72 h. The methanolic solution was then isolated and centrifuged (3000 rpm, 10 min) to separate any suspended bone particulates out of the methanol. The supernatant was then transferred into a clean glass test tube, and evaporated under inert gas at 50°C. Residues were then reconstituted in deionized water (1 mL) and underwent the LLE procedure to effect ketamine extraction from the bone tissues.

Extraction

The pH of aqueous extracts from bone and marrow tissues was adjusted to 8.5–9 by the addition of 50 μL of 0.01 M NaOH. Toluene (5 mL) was added to each sample, and the samples were rotated for 1 h. Following centrifugation (3000 rpm, 10 min), the toluene layer was isolated and combined with 1 mL of 0.1 M H_2SO_4. The mixtures were further rotated on the laboratory rotator for an additional 40 min, vortexed (10 min), and centrifuged (3000 rpm, 10 min). The aqueous layer was isolated and the pH of the aqueous phase was made neutral by the addition of 0.5 M NaOH. The aqueous extracts were then analyzed by ELISA. An internal standard was not used in this extraction. This was to minimize any potential for contributions to the ELISA response through cross-reactivity.

ELISA

Semi-quantitative measurements of ketamine concentration in bone marrow and mineralized bone were made using commercially available ELISA kits (Neogen, Inc., Lexington, KY), as per the manufacturer's instructions. Immunoassay was automated using a ChemWell® 2910 Automated EIA and Chemistry Analyzer purchased from Awareness Technologies (Palm City, FL). The analytical protocol used a microwell plate temperature of 25°C. In each microwell, 20 μL of the aqueous sample solution was combined with 180 μL of diluted enzyme conjugate. The plate was shaken gently for 10 sec and allowed to incubate for 45 min without agitation. The wells were then washed three times with 100 μL of phosphate buffered saline solution (pH 6.5). Following aspiration of the wash solution, 150 μL of K-Blue enzyme substrate (3,3′,5,5′-tetramethyl benzidine (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 red stop solution to each well, followed by gentle agitation for 10 sec. The absorbance of each well was then measured at 630 nm. The concentration dependence of the assay response was evaluated through assay of standard solutions of ketamine prepared in deionized water, both before and after the extraction protocol. Further, the concentration dependence of the assay response was verified by analysis of matrix-matched standards, where drug-free bone or marrow samples were fortified with ketamine at the methanolic incubation stage (bone) or the alkaline homogenization stage (marrow). Standards corresponding to final solution concentrations of 0, 10, 25, 50, 100, and 200 ng/mL ketamine were prepared in this manner.

GC-ECD Analysis of Ketamine and Norketamine

GC-ECD was used to provide qualitative data indicative of the presence of ketamine and norketamine in ELISA-positive samples. The system used was Varian CP-3800 Gas Chromatograph, fitted with a CP-8400 autosampler, a 15 mCI ^{63}Ni electron capture detector, and a CP-Sil 5 CB capillary column (0.25 mm I.D. × 0.25 μm film thickness × 15 m length; Varian®, Palo Alto, CA). Ketamine and norketamine were derivatized using TFAA. Aqueous standard solutions of ketamine and norketamine (0, 10, 25, 50, 100, and 200 ng/mL in 1.0 mL of deionized water) were prepared, and the pH was subsequently adjusted to 8.5–9 with 50 μL of 0.01 M NaOH. Toluene (5 mL) was added and the solutions were rotated for 1 h. The solutions were then transferred to disposable glass vials and the organic layer isolated following centrifugation at 3000 rpm for 10 min. The toluene was then evaporated under inert atmosphere at 50°C and the residue was reconstituted in 500 μL of ethyl acetate. Concentrated TFAA (100 μL) was then added to each sample, and derivatization proceeded in capped vials with heating to 60°C for 30 min. The solution was then evaporated to dryness under inert gas at room temperature. The residues were reconstituted in 300 μL of ethyl acetate. Sample aliquots of 1.0 μL were then analyzed by GC-ECD using a temperature program with initial temperature of 40°C, held for 1 min and subsequently increased to 140°C at a ramp rate of 10°C/min. The temperature was then held at 140°C for 10 min and then brought to 170°C in 2.0°C/min increments. The temperature was held for 10 min and then increased to 250°C at 25°C/min and held for 4 min to end the run. The carrier gas was nitrogen at a constant flow rate of 1.7 mL/min. The injector and detector temperatures were set to 250°C and 300°C, respectively, with the make up flow at the detector set to 25 mL/min.

Qualitative Identification Criteria—GC-ECD

Drug-free samples of marrow and bone were prepared using the extraction and derivatization protocols described above and analyzed by GC-ECD to assess the potential for chromatographic interference.

Extracts of drug-free marrow and bone ($n_{bone} = n_{marrow} = 8$) were derivatized, as were drug-free marrow and bone extracts spiked at various concentrations (ranging from 10 to 200 ng/mL) of ketamine and norketamine. Identification was based on retention time (t_R), where analytes (ketamine, norketamine) were considered detected if the t_R of a given peak was within 2 SD of the mean t_R value measured using matrix-matched standard extracts (where t_R data were collected and averaged over 10 injections, covering a concentration range of 10–200 ng/mL), and the corresponding peak area was greater than the mean area of any peaks meeting the same t_R criteria in drug-free control sample extracts, plus 3 SD. The t_R values (mean ± SD) for ketamine and norketamine were 24.5 ± 0.02 min and 17.1 ± 0.02 min. The limits of detection (LOD) of the GC-ECD assay were *c.* 5 and 20 ng/mL in bone and marrow extracts, respectively, based on a definition of the LOD as the analyte concentration corresponding to a mean signal observed in the appropriate control samples plus 3 SD.

Results

Performance Characteristics of the ELISA Method for Ketamine Detection—Precision, Cross-Reactivity, and Concentration Dependence of Response

The overall precision of this methodology depends on the variability associated with replicate assays of tissue extracts in different microwells (system precision), the variability associated with the extraction (extraction precision) and biological variability associated with distribution of ketamine into the different tissues (e.g., left femoral bone vs. right femoral bone). The precision of replicate analyses (i.e., coefficient of variation) of a given bone extract ranged from 0% to 10% (with respect to absorbance measurements generated from duplicate assays of a given sample extract). In analyses of a given bone type from different limbs within the same animal (e.g., left vs. right epiphyseal fragment) coefficients of variation ranged from 2% to 38%. The precision of replicate analyses of a given marrow sample ranged from 0.1% to 11% (with respect to absorbance measurements generated from duplicate assays of a given sample extract), while the coefficients of variation ranged from 3% to 44% in assays of different marrow samples within the same animal (right bone vs. left).

The efficiency of the extraction was examined through assay of extracted aqueous standard solutions ranging in concentration from 10 to 200 ng/mL and comparison to a standard curve derived from the direct assay of aqueous standards over the same concentration range. Multiple standard curves ($n = 4$) verified the concentration dependence of the assay. Comparison of response from extracted and unextracted aqueous standards to estimate extraction efficiency was initially done by linearization of each data set via a plot of the natural logarithm of absorbance versus that of concentration (i.e., ln A vs. ln C, where R^2 ranged from 0.95 to 0.98). This analysis yielded estimates of extraction efficiency ranging from 70% to 154%. However, due to the nonlinear nature of the ELISA response with respect to analyte concentration, significant error may be introduced to estimations of extraction efficiency in this manner. Thus, direct comparison of absorbance values of extracted and unextracted standards showed coefficients of variation ranging from 4.7% to 24.8%. It must be noted that the variability reflected in these measurements includes contributions from both extraction efficiency as well as from variability of replicate measurements between microwells. Additionally, these data are limited in the information provided about the true efficiency of extraction of drug from mineralized bone tissue. However, while it is not possible to determine the efficiency of drug extraction from the solid bone matrix using this methodology, multiple extractions of single processed bone samples yielded no significant changes in ELISA response beyond the first methanolic extraction and subsequent LLE cycle.

Utility of ELISA for Semi-Quantitative Measurement of Ketamine

To use ELISA as a method for comparison of different skeletal tissue samples for relative sensitivity of ketamine detection, a number of parameters related to assay performance required characterization. First, the response of the assay must be shown to be concentration dependent. Second, the cross-reactivity of the assay to ketamine metabolites and to co-extracted compounds endogenous to the skeletal tissues must be characterized. Finally, the stability of ketamine in the alkaline solution used in bone cleaning and marrow homogenization must be characterized.

Concentration and Dose-Dependence of ELISA Response

Matrix-matched standard solutions of ketamine over the concentration range 0–200 ng/mL were prepared and extracted by the LLE protocol described. These solutions then underwent ELISA for ketamine. Raw absorbance data were then transformed to determine the relative decrease in absorbance, expressed as a percentage of the mean drug-free control sample absorbance (%DA), according to equation (1):

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

where A is the mean absorbance of a given sample and A_{ctrl} is the mean absorbance value of the set of matrix-matched drug-free control tissue extracts. Figure 1 illustrates an example of standard data illustrating the expected concentration-dependent change in %DA measurements generated using this assay.

Decreases in absorbance for tissue extracts derived from drug-positive animals, measured relative to absorbance values derived from the corresponding tissue (i.e., marrow, epiphyseal bone, or diaphyseal bone) from drug-free control animals, were computed for all extracts assayed from animals in groups 1 and 2. Figure 2

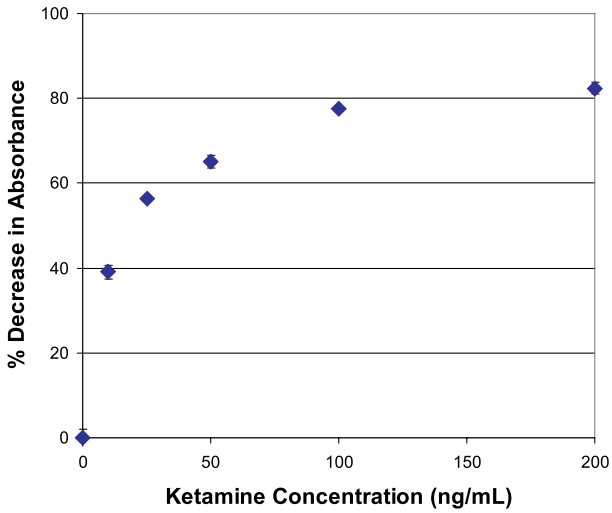

FIG. 1—*Relative decrease in absorbance (%) measured using ELISA method for ketamine as a function of solution ketamine concentration.*

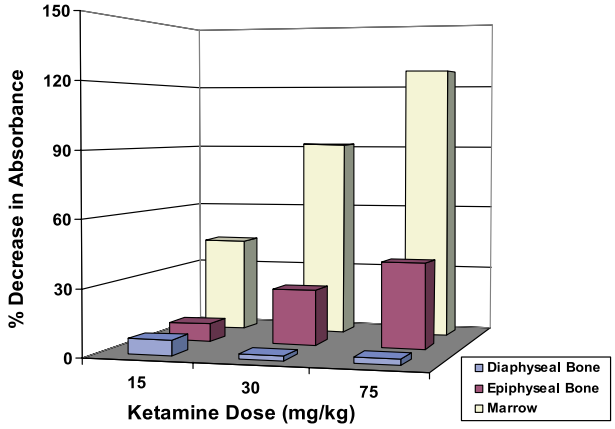

FIG. 2—*Mean relative decrease in absorbance (%) versus administered ketamine dose for animals in group 1 observed in assays of extracts of marrow, epiphyseal bone, and diaphyseal bone.*

presents a plot of mean decrease in absorbance measured in assays of extracts of marrow, epiphyseal bone, and diaphyseal bone, versus ketamine dose for animals in group 1, while Fig. 3 presents a plot of mean decrease in absorbance measured in assays of extracts of marrow, epiphyseal bone, and diaphyseal bone for each animal in group 2. A positive correlation was observed between mean decrease in absorbance and ketamine dose in assays of marrow and epiphyseal bone, but the correlation was poor in assays of diaphyseal bone ($R^2 = 0.87$, marrow; $R^2 = 0.88$, epiphyseal bone; $R^2 = 0.35$, diaphyseal bone). Relative decreases in absorbance observed in assays of extracts of marrow were significantly greater than those of extracts of epiphyseal bone ($p < 0.01$) at all dose levels examined in group 1 as well as those tissues in group 2. Similarly, relative decreases in absorbance observed in assays of extracts of epiphyseal bone were significantly greater than those of extracts of diaphyseal bone ($p < 0.01$) at all dose levels examined in group 1, and in those assays of tissues from group 2.

ELISA Cross-Reactivity Studies—Norketamine

Solutions of norketamine, the primary metabolite of ketamine, were prepared over a wide concentration range to evaluate their cross-reactivity with the ELISA kits. Experimental solutions of norketamine were prepared with concentrations of 100, 1000, and 10,000 ng/mL and were measured against standard ketamine calibration solutions by ELISA (10–200 ng/mL). Additionally, solutions of xylazine were prepared at concentrations of 0.01, 0.1, 1, and 10 mg/mL and measured against standard ketamine calibration solutions by ELISA. The mean cross-reactivity of norketamine was determined to be *c.* 4.2%. The mean cross-reactivity of xylazine was determined to be *c.* 0.01%. Hence, xylazine was considered to be essentially nonreactive with Neogen ELISA kits for ketamine.

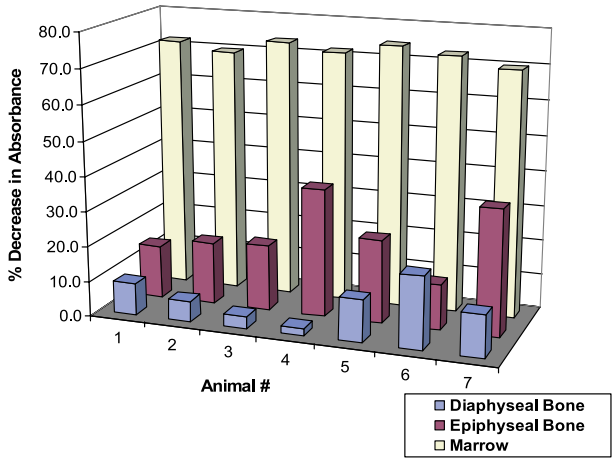

FIG. 3—*Mean relative decrease in absorbance (%) for each animal in group 2 observed in assays of extracts of marrow, epiphyseal bone, and diaphyseal bone. Data shown correspond to mean of values obtained.*

ELISA Cross-Reactivity Studies—Endogenous Compounds Within the Skeletal Tissue Matrix

A series of control tissue samples derived from drug-free animals underwent the extraction and analysis procedures along with those derived from drug-positive animals. In some cases these samples did generate a measurable change in ELISA response relative to a sample of pure deionized water, which suggests that compounds endogenous to skeletal tissues are retained in the extraction and undergo some apparent cross-reactivity with the immobilized antibodies. When the assay response to drug-free extracts from epiphyseal and diaphyseal bone were compared, no significant differences in response were measured between the two groups ($p = 0.6$), suggesting that there were no site-dependent differences in cross-reactivity to endogenous compounds within the bone matrix.

Effect of Solution pH on ELISA Response to Ketamine

Nine sets of ketamine-spiked deionized water at 10 and 100 ng/mL were fixed to a pH of 8.5–9.0 by the addition of 50 μL of 0.01 M NaOH to each. Each set of solutions was then allowed to sit for time intervals of 0, 0.5, 1, 2, 4.5, 6.5, 24, 48, and 72 h at 21°C before being neutralized to pH 6.5 with 0.05 M H_2SO_4. The effect of the alkaline treatment was evaluated by ELISA for each time interval. Increasing the exposure time of ketamine to elevated pH had no significant effect on the ELISA response to the standard solutions. Measured relative standard deviations of response were 8.7% and 9%, respectively.

Binary Test Sensitivity of Ketamine Detection in Marrow, Epiphyseal Bone, and Diaphyseal Bone

The use of ELISA as a screen for the presence of ketamine 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

$$\text{Sensitivity(S)} = 100\% \times \frac{\text{TP}}{\text{FN} + \text{TP}} \qquad (2)$$

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). In this work, ketamine was considered detected in a given sample if the ELISA response (i.e., absorbance at 630 nm) was less than the mean absorbance of drug-free extracts of the corresponding tissue, minus 3S. The ELISA response for each sample assayed was compared against the appropriate control group (i.e., marrow, epiphyseal bone, or diaphyseal bone) and a designation of

detected or *not detected* was assigned. As such, the binary classification sensitivity of the ELISA assay was determined for the tissues examined in groups 1 and 2, and values are presented in Table 1 according to ketamine dose and the type of tissue assayed (i.e., marrow, epiphyseal bone, or diaphyseal bone).

GC-ECD Analysis of Bone and Bone Marrow Extracts

Subsequent to analysis by ELISA, extracts of bone and marrow samples were qualitatively analyzed by GC-ECD to provide supportive data in the identification of ketamine and norketamine. This analysis was only qualitative, as an internal standard was not added to the tissue sample prior extraction to minimize the extent of cross-reactivity contributions to the ELISA signal. For GC-ECD analysis of a given sample extract, attribution of a given peak to ketamine or norketamine required satisfaction of two criteria. Peaks were only attributed to ketamine or norketamine if (i) the measured retention time (t_R) was within 2 SD of the mean t_R value measured in replicate analyses of drug-free matrix (bone or marrow) spiked with ketamine and/or norketamine prior to extraction and (ii) if the measured peak area was greater than the mean area plus 3 SD of any integrated peaks in all drug free control samples which met the t_R criteria described in (i). The number of extracts in each experimental condition (i.e., dose, tissue type sampled) in which ketamine or norketamine were detected are listed in Table 2.

TABLE 1—*Relative decrease in absorbance values derived from ELISA analysis of marrow (MRW), diaphyseal bone (DIA), and epiphyseal bone (EPI) extracts from drug-positive tissues.*

Group	Dose (mg/kg)	Tissue Sampled	% Decrease in Absorbance
1	15	MRW	Min: −6.3 Max: +87.6 Mean ± SD: 43 ± 35
1	15	DIA	Min: −8.7 Max: +19.3 Mean ± SD: 6.9 ± 11.5
1	15	EPI	Min:−9.5 Max: +30.4 Mean ± SD: 8.2 ± 15.4
1	30	MRW	Min: +65.9 Max: +109.9 Mean ± SD: 91 ± 16
1	30	DIA	Min:−4.7 Max: +7.0 Mean ± SD: 2.0 ± 4.9
1	30	EPI	Min: +21.3 Max: +27.4 Mean ± SD: 25.3 ± 2.3
1	75	MRW	Min: +121.5 Max: +140.0 Mean ± SD: 127 ± 8
1	75	DIA	Min: −2.0 Max: +4.9 Mean ± SD: 2.7 ± 5.7
1	75	EPI	Min: +35.7 Max: +41.5 Mean ± SD: 38.9 ± 2.1
2	75	MRW	Min: +69.7 Max: +74.5 Mean ± SD: 72.0 ± 1.8
2	75	DIA	Min: −6.3 Max: +8.3 Mean ± SD: 9.6 ± 8.9
2	75	EPI	Min: +2.6 Max: +61.2 Mean ± SD: 25.3 ± 15.4

TABLE 2—*Binary classification sensitivity of ELISA analysis of marrow (MRW), diaphyseal bone (DIA), and epiphyseal bone (EPI) extracts from drug-positive tissues.*

Group	Dose (mg/kg)	Tissue Sampled	Number of Samples With Positive ELISA Results	Total Number of Samples Assayed	ELISA Sensitivity, S (%)	Number of Samples With Positive GC-ECD Results
1	15	MRW	3	4	75	1
1	15	DIA	2	4	50	1
1	15	EPI	1	4	25	0
1	30	MRW	4	4	100	3
1	30	DIA	0	4	0	2
1	30	EPI	4	4	100	4
1	75	MRW	4	4	100	4
1	75	DIA	0	4	0	1
1	75	EPI	4	4	100	4
2	75	MRW	13	13	100	13
2	75	DIA	3	13	23	4
2	75	EPI	10	13	77	8

Analysis of tissue extracts from group 1 by GC-ECD showed that there were no significant peaks detectable in the negative control marrow that could have co-eluted with ketamine or norketamine based on the t_R values provided. In three of the four 15 mg/kg marrow samples and one of four samples in the 30 mg/kg dose group, ketamine was not detected. The positive correlation of detected ketamine with dose was still observed with the results from the GC-ECD (R^2 = 0.86), but the variation observed for each subpopulation was markedly increased compared with ELISA results. Examination of GC-ECD data corresponding to extracted standards also displayed a concentration-dependent response with variable linearity (R^2 = 0.89–0.99). This was likely due, at least in part, to the lack of internal standard.

An example set of GC-ECD chromatographic data corresponding to tissues from group 2 is shown in Figs. 4 and 5. In Fig. 4*A*, corresponding to the analysis of an extract of epiphyseal bone from a drug-positive animal, the peaks labeled as ketamine and norketamine satisfied the qualitative identification criteria. In Fig. 4*B*,

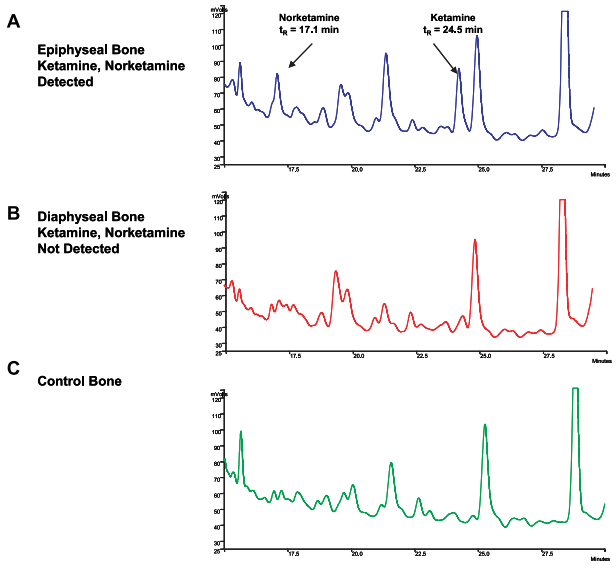

FIG. 4—*GC-ECD chromatograms corresponding to derivatized extracts of (A) epiphyseal bone, (B) diaphyseal bone of a single rat given ketamine (75 mg/kg i.p., group 2), and (C) epiphyseal bone of a single drug-free control rat.*

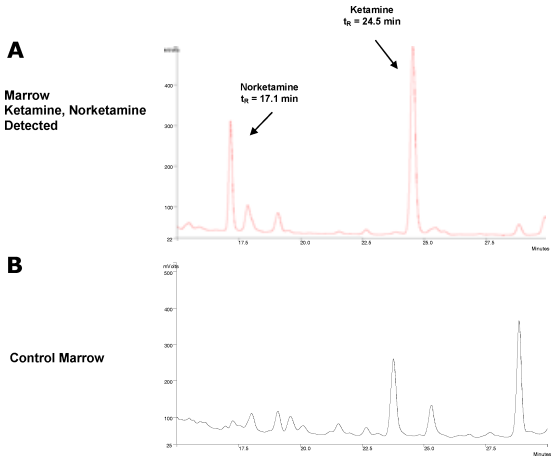

FIG. 5—*GC-ECD chromatograms corresponding to derivatized extracts of (A) marrow of a single rat given ketamine (75 mg/kg i.p., group 2) (B) marrow of a single drug-free control rat.*

corresponding to the analysis of an extract of diaphyseal bone from the same femur, the identification criteria were not satisfied and both ketamine and norketamine were considered not detected in this sample. Figure 4*C* corresponds to the analysis of a drug-free control epiphyseal bone extract and is provided for comparison. Similarly, Figs. 5*A* and 5*B* correspond to the GC-ECD analysis of extracts of marrow from a drug-positive animal and control animal, respectively.

Examination of the GC-ECD data for the two groups demonstrated that ketamine peak areas corresponding to epiphyseal bone extracts were significantly different than those of the diaphyseal fragments for group 2 ($p < 0.01$) but not for group 1 (75 mg/kg dose). The ratio of peak areas (epiphyseal:diaphyseal) ranged from 0.79 to 6.9 with a mean value of 1.6.

Discussion

Bone Cleaning and Sample Preparation

The data shown in Table 1 illustrate that there are significant differences in relative decrease in absorbance observed in assays of extracts of mineralized bone and bone marrow. This pattern of drug distribution between marrow and mineralized bone has been noted in other reports with respect to other drugs, as well (10). This highlights the need to develop stringent methods for separation of marrow from mineralized bone so as to independently assess the value of a particular tissue type in detection of a given drug, as residual marrow or other soft tissue on a bone fragment may result in the artificial elevation of measured drug concentrations. This is particularly important and challenging in preparing epiphyseal bone fragments derived from rat femora, where the viscous marrow fills the dense network of tightly packed trabeculae. The method chosen to achieve this was based on ultrasonication of the bone fragments in a solution containing 0.25 M NaCl and 0.25 M NaOH. This solution was chosen on the basis that the high pH would not result in the digestion of the mineral bone matrix, but would dissolve the marrow. Recognizing the potential for inadvertent extraction of drug from the mineralized bone matrix during this step, as well as the potential for overestimation of relative decrease in absorbance observed in assays of extracts of mineralized bone due to contamination by residual bone marrow, the addition of salt was intended to maintain a high ionic strength to reduce the potential for diffusion of drug/metabolite from the mineral bone matrix in solution. Assays of ketamine solutions exposed to this alkaline solution for up to 72 h showed no significant alteration in assay response relative to controls, suggesting that this treatment does not result in significant alterations to the immunochemical reactivity of ketamine.

Following the treatment of bone fragments in the alkaline solution, the cleanliness of the bone fragments was assessed by means of microscopic observation. Photographs of bone fragments before and after the cleaning process are presented in Fig. 6. By manually cracking bone fragments and examining their microscopic appearance, the absence of visible traces of residual marrow was ensured.

Extraction of drug from bone samples has been done by incubation of bone fragments in methanol (6,8), and by digestion of bone fragments in strong acid (e.g., 3 N HNO_3) (5). Pulverization and subsequent methanolic extraction was chosen for this work as it is a gentle extraction method, although it may arguably be less efficient than one based on digestion in acidic media. Subsequent to drug extraction into methanol, extracts then underwent a standard LLE for further sample clean-up. Although the combination of significant sample clean-up prior to analysis of drug extracts by immunoassay methods is not a common practice in forensic applications, it has been reported in the literature as a means to improve sensitivity and time window of detection of some drugs, including ketamine (22). This methodology has been valuable in this work in reducing background signal and thereby improving sensitivity of detection in extracts of mineralized bone fragments, where estimated ketamine equivalent concentrations typically ranged from 5 to 30 ng/mL.

Suitability of ELISA for Detection of Ketamine Exposure in Skeletal Tissues: Immunoassay Selectivity and Performance

The use of ELISA in the comparison of the relative sensitivity of detection of ketamine exposure in different sample matrices requires that the analytical response must be at least semi-quantitative with respect to drug concentration, and that differences in assay response to extracts from different matrices are not due to endogenous components. Despite the fact that ELISA can provide an analytical response that is dependent upon analyte concentration, the use of immunoassay for semi-quantitative or quantitative analysis is uncommon in forensic settings, primarily due to the cross-reactivity with various compounds in biological samples and the unknown composition of forensic samples. In this application, the use of immunoassay for semi-quantitative work is less problematic due to the ability to control the drug and nutritional intake of experimental animals. In such cases, cross-reactivity with endogenous compounds can be accounted for to a large extent through monitoring of analytical response in matrix-matched drug-free animal tissues. In this work, a number of extracts of drug-free bone and marrow tissues derived from multiple animals were assayed and used as the baseline against which the ELISA response of drug-positive tissues were measured. The data collected to date indicate that drug-free tissue extracts may yield a different ELISA response than samples containing pure solvent (e.g., deionized water), indicating the potential for some cross-reactivity between endogenous compounds in bone and marrow with the antibodies in the ELISA microwells. However, the data presented here show positive correlation between the ketamine dose administered and the relative decrease in absorbance observed with marrow and epiphyseal bone extracts. Furthermore, analysis of bone tissues derived from drug-free animals showed no significant differences in ELISA response to extracts of diaphyseal and epiphyseal bone, indicating that any differences observed in ELISA response between those two tissue types within a given animal or across a

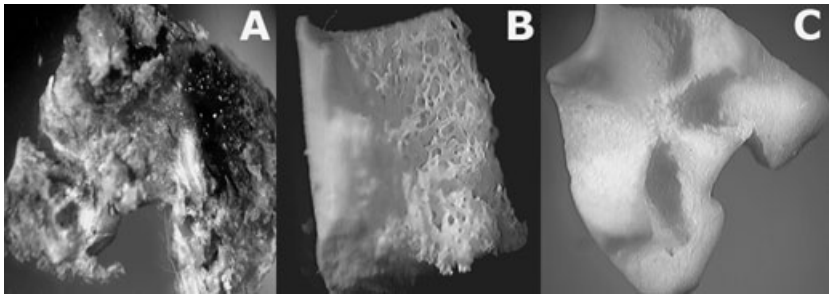

FIG. 6—*(A) Uncleaned trabecular bone, (B) cleaned trabecular epiphyseal bone surface, and (C) cleaned cortical diaphyseal bone surface. Graphics recorded to assess the efficacy of the employed ultrasonication cleaning procedure.*

group of animals is more reflective of differences in drug distribution into those tissues and/or efficiency of ketamine extraction from those tissues.

The potential for cross-reactivity of immobilized antibodies with co-administered compounds and drug metabolites also exists, creating the potential for false positive signal generation. Consequently, the selectivity of the ELISA system used here was assessed with respect to cross-reactivity of the immobilized antibodies with norketamine and xylazine. In both cases, aqueous solutions containing 100, 1000, or 10,000 ng/mL were assayed using this ELISA system. With respect to norketamine, the mean cross-reactivity with antibodies to ketamine was determined to be *c*. 4.2%. Even in the analysis of marrow samples, where drug concentrations were significant, ratios of peak area of ketamine relative to those of norketamine ranged from 1.0 to 7.5. Furthermore, norketamine detection is substantially more sensitive (*c*. two- to threefold) than ketamine detection by the GC-ECD method described here, presumably due to greater efficiency of derivatization. This supports the assertion that norketamine concentrations were substantially lower than ketamine concentrations in the samples examined here. Coupled with the low cross-reactivity of norketamine with the ELISA kits used here, this suggests that the contributions of norketamine to the ELISA signal may be generally negligible under these conditions. Nonetheless, contributions to ELISA response from ketamine metabolites also constitute an indication of ketamine exposure, and are therefore valuable.

Implications of Detection of Ketamine in Marrow, Epiphyseal, and Diaphyseal Bone

The results suggest that acute ketamine exposure can be reproducibly detected by ELISA in marrow and mineralized epiphyseal tissue, while measurements using diaphyseal portions were less reliable. The data in Figs. 2 and 3 and Table 1 show that the sensitivity of ketamine detection is greatest for marrow, and is greater for epiphyseal bone in comparison with diaphyseal bone, when estimated via both the relative decrease in absorbance and the binary classification test sensitivity of the ELISA method. This is consistent with expectations based on the high lipid content of the marrow as well as data for other drugs in published reports (8,10). These data may indicate that epiphyseal bone ketamine equivalent concentrations were significantly higher than the corresponding diaphyseal bone ketamine equivalent concentrations. This is reasonable, as compounds within the marrow may diffuse through cannaliculi in the trabecular projections. Given the high surface area exposed to marrow and relatively low thickness of the bone trabeculae, significant diffusion into the mineralized matrix seems more likely in epiphyseal bone than in diaphyseal bone. Although the accuracy of the GC-ECD analysis may be limited by the lack of an internal standard, peak area ratio data for these tissues support this general trend.

Lien et al., in a study of the disposition of tamoxifen in human and rat tissues, reported detection of tamoxifen and metabolites in cortical and trabecular bone of a single patient (23). In that study, the authors indicated that trabecular bone tamoxifen concentrations were higher than cortical bone tamoxifen concentrations and suggested that this discrepancy may have been due to variation in extraction efficiency from the two tissues. The mechanism behind the more sensitive assay response observed in assays of epiphyseal bone extracts relative to those of diaphyseal bone extracts cannot be elucidated based on the data presented here. However, these results suggest that analysis of epiphyseal bone fragments may be advantageous in terms of providing improved probability of drug detection following acute exposure.

In this work, the entirety of each of fragment (i.e., epiphyseal or diaphyseal) was processed for drug extraction, as opposed to weighing a targeted mass of each type of bone for extraction after cleaning and pulverization. As it was initially unknown if there was significant spatial heterogeneity with respect to bone drug concentration, this methodology ensured that significant portions of drug-positive bone were not excluded in sampling. The data presented here raise the possibility that there may be significantly greater ketamine concentrations in trabecular (epiphyseal) bone than in cortical (diaphyseal) bone.

These data may have significant implications for drug screening and analysis in human tissues. If the site-dependent ELISA sensitivity is reflective of heterogeneous drug distribution in a given bone, then the possibility exists that partial bone sampling (as would likely be done in analyses of human bone tissues) may yield no detectable drug concentrations even when a drug is present. 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 to minimize the risk of false negatives.

Conclusions and Summary

These data have shown that ELISA may be a valuable tool in screening skeletal tissue samples for drugs of abuse. Furthermore, marrow may serve as a significant depot for ketamine, as it has been shown to be for a number of other drugs (5,10,11,14). These preliminary results indicate 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. The effects of various environmental factors (e.g., burial, humidity, temperature) were not examined and will be the

focus of future work. The examination of other drugs of forensic relevance with different chemical and pharmacological properties will also be undertaken to determine if similar distribution properties are observed.

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Additional information and reprint requests:
James Watterson, Ph.D.
Department of Forensic Science
Laurentian University
935 Ramsey Lake Rd.
Sudbury, ON
Canada P3E 2C6
E-mail: jwatterson@laurentian.ca