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Detection of Acute Diazepam Exposure in Bone and Marrow: Influence of Tissue Type and the Dose-Death Interval on Sensitivity of Detection by ELISA with Liquid Chromatography Tandem Mass Spectrometry Confirmation*

ABSTRACT: Enzyme-linked immunosorbent assay (ELISA) and liquid chromatography tandem mass spectrometry (LC/MS/MS) were used to detect diazepam exposure in skeletal tissues of rats (n = 15) given diazepam acutely (20 mg/kg, i.p.), and killed at various times postdose. Marrow, epiphyseal, and diaphyseal bone were isolated from extracted femora. Bone was cleaned, ground, and incubated in methanol. Marrow underwent ultrasonic homogenization. Extracts and homogenates were diluted in phosphate buffer, and then underwent solid-phase extraction and ELISA. Relative sensitivity of detection was examined in terms of relative decrease in absorbance (ELISA) and binary classification sensitivity (ELISA and LC/MS/MS). Overall, the data showed differences in relative sensitivity of detection of diazepam exposure in different tissue types (marrow > epiphyseal bone > diaphyseal bone), which is suggestive of heterogenous distribution in these tissues, and a decreasing sensitivity with increasing dose-death interval. Thus, the tissue type sampled and dose-death interval may contribute to the probability of detection of diazepam exposure in skeletal tissues.

KEYWORDS: forensic science, bone, marrow, drugs, diazepam, immunoassay, liquid chromatography, mass spectrometry

A number of studies have been published in recent years describing drug detection in skeletal tissues (1–6). The majority of work in this field has involved the use of animal tissues in analytical method development and basic drug distribution research, since the use of human tissues is problematic due to the obvious ethical constraints associated with controlled skeletal tissue sampling, and difficulties with the use of autopsy tissues including poorly characterized drug use history and the potential for legal restrictions in some jurisdictions. Consequently, the lack of basic research on drug disposition in human skeletal tissues virtually precludes the ability to associate a measured drug concentration in a bone or marrow sample with toxicity in forensic casework.

Still, valuable forensic information may be gleaned in some cases by means of qualitative or semi-quantitative toxicological analyses of skeletal tissues. For example, the identification of the presence of a drug that was not expected in a particular sample may provide investigative direction that may not have otherwise existed. Through the use of qualitative or semi-quantitative measurements, experimental data highlighting the effects of tissue type (i.e., marrow vs. bone), sites of sampling (e.g., vertebra vs. femur, trabecular vs. cortical bone) and the delay between drug administration and death (i.e., the *dose-death interval*) are valuable for optimizing the probability of drug detection and understanding the implications and limitations of the analytical results.

In recent work (7), we illustrated the utility of enzyme-linked immunosorbent assay (ELISA) and gas chromatography (GC) in the detection of ketamine exposure in assays of extracts of marrow and mineralized bone from rats acutely exposed to the drug. The sensitivity of the ELISA response was shown to be dependent upon the dose administered and the type of tissue examined (marrow, epiphyseal bone, or diaphyseal bone). Here, we discuss the extension of this methodology to the detection of diazepam exposure *via* assay of extracts of skeletal tissues in rats given acute doses of diazepam. Diazepam is a benzodiazepine used as a sedative-hypnotic and anticonvulsant. It is a central nervous system (CNS) depressant whose effects are compounded when coadministered with other CNS depressants, such as alcohol. Its depressant effects, including drowsiness, stupor, loss of consciousness, and amnesia, implicate its potential utility in drug-facilitated sexual assault.

The detection of benzodiazepines in skeletal tissues has been reported in the literature. In one of the first reports of drug detection in mineralized bone, Gorczynski and Melbye (5) reported the use of ELISA in the detection of benzodiazepines in marrow and mineralized bone of mice exposed to the drugs under both acute and chronic conditions. While the authors reported that investigations had been performed using diazepam, lorazepam, midazolam, and triazolam, only those data related to midazolam administration

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were discussed in the report as the assay used displayed the greatest sensitivity of response toward this drug. In that work, midazolam exposure was detectable in the marrow of mice receiving midazolam both acutely and under chronic dose administration, while it was detectable in the bone only in cases of chronic drug administration.

Winek et al. (8) reported the use of GC with electron capture detection in the detection of flurazepam in the marrow of rabbits given the drug acutely. In that work, marrow:plasma flurazepam concentration ratios ranged from 18 to 100, with a mean value of 34.5. This finding highlighted the extent to which lipophilic drugs may partition into lipid-rich marrow. In these reports, however, the effect of the time elapsed between drug administration and death (i.e., the dose-death interval) was not examined. This issue is of forensic relevance as it directly affects the implications of a positive result in a skeletal tissue drug analysis, in terms of a potential time window for drug exposure under a given set of drug administration conditions.

In this work, we illustrate the detection of acute diazepam exposure in both bone and marrow of acutely exposed rats. Analysis was performed using automated solid-phase extraction (SPE) in combination with ELISA in a microwell format, and qualitative confirmation by liquid chromatography tandem mass spectrometry (LC/MS/MS). The purpose of this work was to examine the effect of the tissue type sampled and the dose-death interval on the sensitivity of the assay response and to illustrate the applicability of techniques routinely used in the forensic toxicology laboratory to drug detection in skeletal tissues.

Methods

Chemicals

Methanol used in drug extraction was HPLC grade and was obtained 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 were 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 7 days to acclimatize to their conditions. Adult male Wistar rats (Charles River Laboratories, St. Constant, QC, Canada) were housed in groups of three with Harlan Teklad ¼″ 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.

Adult male Wistar rats were given 0 ($n = 4$) or 20 mg/kg ($n = 15$) diazepam (i.p.) and killed within 20 min, 60 min, or 180 min with CO_2 gas. The delay periods between drug administration and killing were chosen on the basis of the half-life of diazepam in rats, which has been measured to range from 53 to 106 min, with a mean value of 72 min (9). Hind legs 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, while 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 1 mL 0.25 M NaOH/0.25 M NaCl with ultrasonication. Homogenized solutions were then diluted in 2 mL phosphate buffer (0.5 M, pH 5.5). The resultant solutions then underwent SPE as described below.

Bone Preparation

After tissues adhering to the surface of the bones had been removed by scraping with a scalpel and 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 and cleaned by ultrasonication for *c*. 60 min. Once cleaned, bone fragments were rinsed with deionized water (dH_2O) and methanol and dried under argon at 50°C. Dried fragments were examined by visible microscopy as described in earlier work (7). The medullary cavities were then flushed with this washing solution and then the samples underwent further ultrasonication for 30 min. The washed solution was discarded and the bones were sequentially rinsed with dH_2O and methanol, and then dried under a steady flow of argon at 50°C. The bones were then grounded 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 3 mL phosphate buffer (0.1 M, pH 6) and underwent SPE.

Solid-Phase Extraction

Marrow homogenates and reconstituted bone extracts underwent SPE using Oasis HLB columns, 3 cc/60 mg (Waters Inc., Milford, MA) using a Gilson ASPEC XL-274 Automated SPE instrument (Middleton, WI). Columns were conditioned with 2 mL methanol and 2 mL dH_2O. Samples were loaded (2.5 mL) at a flow rate of 0.1 mL/min, and columns were sequentially washed with 5:95 methanol:dH_2O (2 mL), followed by 2% acetic acid in 25:75 methanol:dH_2O (2 mL). Methanol (2 mL) was used as the elution solvent. All eluted fractions were evaporated to dryness under argon (50°C) and subsequently reconstituted in dH_2O (1 mL).

Enzyme-Linked Immunosorbent Assay

Immunoassay of diazepam and metabolites in extracts of marrow and mineralized bone were made using commercially available ELISA kits (Immunalysis Corp., Pomona, CA), as per the manufacturer's 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 sec and allowed to incubate for 60 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 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 sec. The absorbance of each well was then measured at 450 nm.

Liquid Chromatography Tandem Mass Spectrometry

All samples with positive ELISA results, measured relative to matrix-matched drug-free tissues, 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 100 ng/mL internal standard (diazepam-D_5, oxazepam-D_5). An Agilent 1200 Series liquid chromatograph pump coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA), operating in positive electrospray ionization mode, was used for analysis. Separation was achieved using a Zorbax Eclipse XDB C18 column (Agilent Technologies) (4.6 mm × 50 mm × 1.8 mm), held at 35°C and 5 μL injection volume. Separation was isocratic, with a mobile phase consisting of 20 mM ammonium acetate (Solvent A, pH 6.4) and acetonitrile (Solvent B) in a 1:1 (v/v) mixture. The initial flow rate was 0.2 mL/min, held for 6.5 min. Flow was then increased to 1 mL/min, and at 8 min, decreased back to 0.2 mL. The total run time was 10 min, with a 4.5 min postrun equilibration period. The mass spectrometer capillary voltage was set to 4500 V and used an initial nebulizer pressure of 15 psi, which was later raised to 50 psi for improved signal. The nitrogen gas flow rate and temperature were 6 L/min and 300°C, respectively. Mass spectrometry was carried out in multiple reaction monitoring mode. The precursor/fragment ions were 285/257 (222) for diazepam, 271/140 (165) for nordiazepam, and 287/241 (269) for oxazepam, where the values in parentheses represent qualifying product ions. The optimized fragmentation voltages for these transitions were 160 V (diazepam and nordiazepam) and 120 V (oxazepam), and the collision energies were 30, 30, and 20 V, for diazepam, nordiazepam, and oxazepam, respectively. Under these conditions, the retention times (t_R) for diazepam, nordiazepam, and oxazepam were 5.8, 3.5, and 2.1 min, respectively. A drug was considered to be identified when measured t_R 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 to be considered positive. The limit of detection of the LC/MS/MS assay was 0.2 ng/mL.

Results

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

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 sample ranged from 0.9% to 13%. In this work, equal volumes of reconstituted marrow extracts from right and left limbs were combined to generate an assay response corresponding to the average of the two marrow samples, as variability between marrow samples from different limbs was the largest source of variability observed in our earlier work (7).

Due to the nonlinearity of the ELISA response with respect to diazepam concentration, extraction efficiency cannot be accurately determined using this method. To examine the effect of SPE on the ELISA response, absorbance values of extracted and unextracted standards (diazepam) at a given concentration level over the range of 5–200 ng/mL were measured. The coefficient of variation between the absorbance values of the extracted and unextracted standards was used to indicate the effect of extraction of the measured ELISA response. These analyses showed coefficients of variation ranging from 3.5% to 25.3% (diazepam), 2.5% to 42.3% (nordiazepam), and 3.5% to 51.9% (oxazepam). Both imperfect extraction efficiency and imprecision of replicate measurements between microwells contribute to this observed variability.

Concentration Dependence of ELISA Response

Standard solutions of diazepam, nordiazepam, and oxazepam were prepared (0–200 ng/mL) and extracted by the SPE protocol described. Extracts were then assayed by ELISA. As described previously (7), 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 Eq. (1):

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

where A is the mean absorbance of a given sample and A_{ctrl} is the mean absorbance value of a set of matrix-matched, drug-free control tissue extracts. Figure 1 illustrates the concentration-dependent %DA measurements as a function of diazepam, nordiazepam, and oxazepam concentration using this assay.

ELISA Cross-Reactivity Studies

As was carried out in our earlier work (7), a series of control tissue samples derived from drug-free animals underwent the

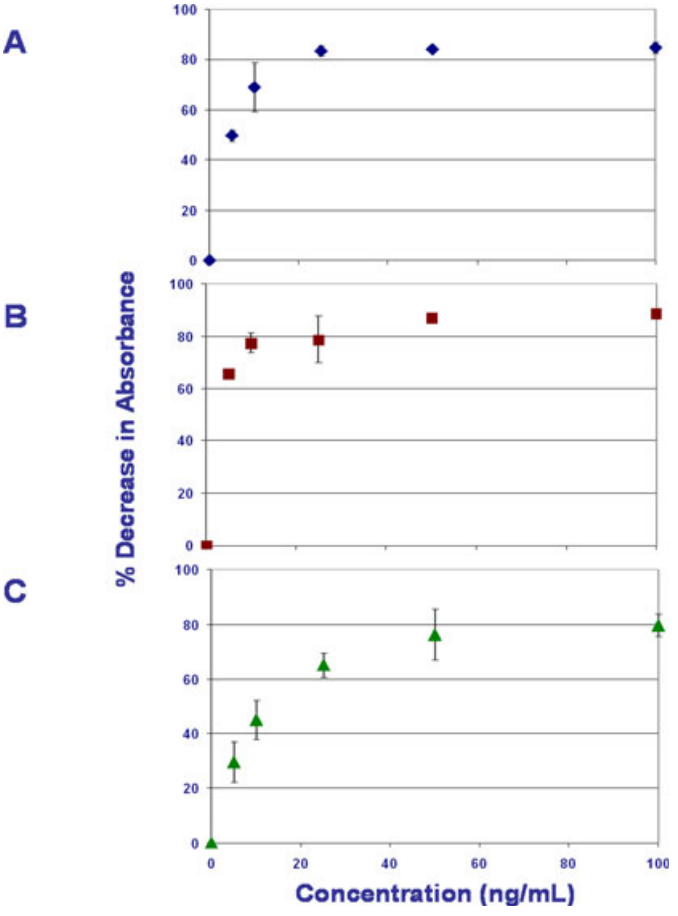

FIG. 1—*Relative decrease in absorbance (%) measured using ELISA method as a function of solution diazepam (A), nordiazepam (B), and oxazepam (C) concentration following solid-phase extraction.*

extraction and analysis procedures along with those derived from drug-positive animals. Comparison of assay response to drug-free extracts from epiphyseal and diaphyseal bone showed no significant differences in response between the two groups ($n = 4$, $p > 0.05$), despite significant differences in mass of the pooled epiphyseal versus pooled diaphyseal bone fragments.

The data shown in Fig. 1 shows that the response of the Immunalysis ELISA kits was significant with respect to the diazepam metabolites, nordiazepam and oxazepam. In these experiments, nordiazepam and oxazepam solutions (5–200 ng/mL) generated %DA values ranging from 66% to 89% (nordiazepam) and 48% to 89% (oxazepam).

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 (Fig. 2*A*) and the corresponding mean %DA values measured in each experimental condition (Fig. 2*B*). Relative decreases in absorbance observed in assays of extracts of marrow differed significantly from those of extracts of epiphyseal bone ($n = 5$, $p < 0.05$) at the 20 and 180 min dose-death intervals, but not at the 60 min dose-death interval. There was no significant difference between the absorbance values of the control and drug positive diaphyseal bone tissue extracts at any of the dose-death intervals examined, so the %DA values were assigned a value of 0 for each dose-death interval shown in Fig. 2.

The effect of the dose-death interval on the observed %DA values for a given tissue type was also examined. For marrow and epiphyseal bone extracts, within-tissue comparison showed that %DA values at the 180 min dose-death interval were significantly different from those at both the 20 min and 60 min dose-death intervals, respectively ($n = 5$, $p < 0.05$), but the %DA values at the 20 and 60 min dose-death intervals did not differ significantly from each other. For diaphyseal extracts, there were no significant differences between %DA values over any of the three dose-death intervals examined. Measured %DA values were negatively correlated with dose-death interval, with correlation coefficients (r) of −0.87 and −0.79 for extracts of marrow and epiphyseal bone, respectively.

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

As was shown in earlier work (7), the use of ELISA as a screen for diazepam exposure in a given skeletal tissue extract was examined through the use of a binary classification test model, where drug exposure was considered to be detected if the ELISA response varied significantly relative to those of the matrix-matched control samples. As such, the binary classification test sensitivity (S) was 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 diazepam in drug-positive tissues) and FN represents the number of false negative detections (i.e., cases where the ELISA response to extracts of tissues from drug-positive animals did not differ significantly from those of drug-free controls). In this work, diazepam exposure 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. By this definition, the limits of detection for diazepam, nordiazepam, and oxazepam in extracted samples were *c.* 2, 0.5, and 5 ng/mL, respectively. 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. The results are summarized in Table 1.

LC/MS/MS Analysis of Skeletal Tissue Extracts

Those samples for which diazepam exposure was considered detected by the ELISA method according to the criteria discussed above underwent confirmatory analysis by LC/MS/MS. Diazepam

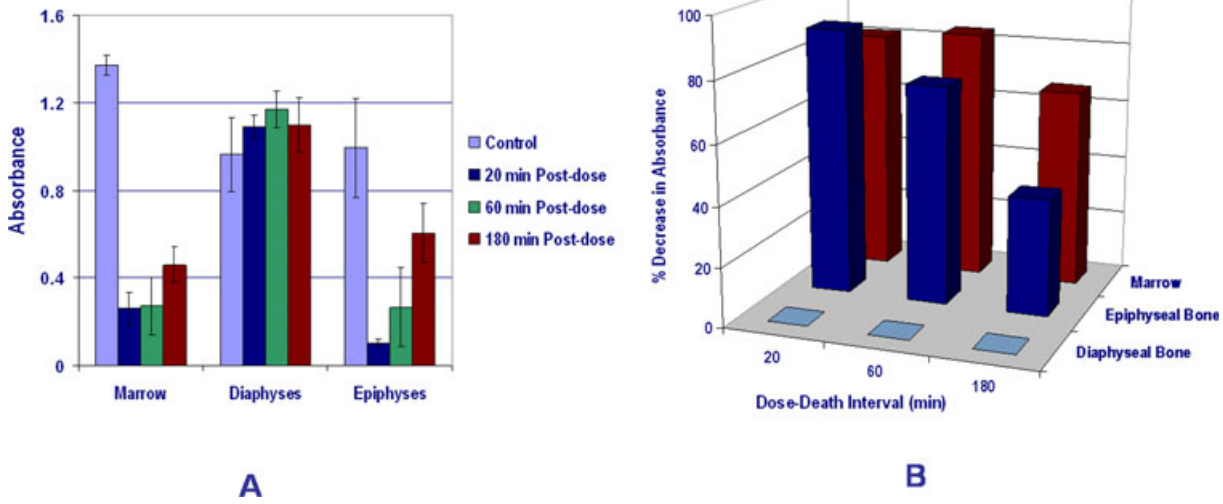

FIG. 2—*(A) Mean absorbance values for extracts of each tissue type, over the dose-death intervals examined; (B) Mean relative decrease in absorbance (%) for each dose-death interval examined in assays of extracts of marrow, epiphyseal bone, and diaphyseal bone.*

TABLE 1—*Relative decrease in absorbance (%DA), binary classification sensitivity of ELISA, and frequency of detection of diazepam, nordiazepam, and oxazepam in LC/MS/MS analysis of marrow, diaphyseal bone, and epiphyseal bone extracts from drug-positive tissues.*

Tissue Sampled	Dose-Death Interval (min)	Relative Decrease in Absorbance (%DA) Mean ± SD (Range)	Percentage Samples Positive by ELISA	Percentage Samples with Diazepam Detected by LC/MS/MS	Percentage Samples with Nordiazepam Detected by LC/MS/MS	Percentage Samples with Oxazepam Detected by LC/MS/MS
Marrow	Control	n/a	n/a	0	0	0
	20	81 ± 5 (75.2–87.5)	100	100	40	80
	60	84 ± 4 (79.1–89.1)	100	100	0	60
	180	67 ± 6 (60.3–76.1)	100	100	0	0
Epiphyseal bone	Control	n/a	n/a	0	0	0
	20	90 ± 2 (88.3–92.6)	100	100	0	0
	60	73 ± 18 (42.6–86.4)	100	100	0	0
	180	39 ± 14 (23.0–52.0)	60	100	0	0
Diaphyseal bone	Control	n/a*	n/a	n/a	n/a	n/a
	20	0*	0	n/a	n/a	n/a
	60	0*	0	n/a	n/a	n/a
	180	0*	0	n/a	n/a	n/a

*The %DA value of a given sample was assigned a value of zero if the %DAsample ≤ %DACTRL + 3SD. ELISA, enzyme-linked immunosorbent assay; LC/MS/MS, liquid chromatography tandem mass spectrometry.

was detected by LC/MS/MS in all of the marrow and epiphyseal bone sample extracts examined, over all of the dose-death intervals investigated. Nordiazepam was detected in 40% of the marrow extracts at the 20 min dose-death interval, but not detected in any other tissue extract examined. Oxazepam was detected in 80% of the marrow extracts at the 20 min dose-death interval, and 60% of the marrow extracts at the 60 min dose-death interval, but not detected in any other tissue extract examined. These data are summarized in Table 1.

Discussion

Value and Limitations of Experimental Approach

The purpose of this work was to examine the utility of ELISA in the detection of diazepam exposure in skeletal tissues. ELISA is a sensitive methodology that is widely used in forensic laboratories, as it has proven very reliable for the screening of forensic samples to minimize the workload in more complex and demanding analyses by other methodologies (e.g., GC/MS and LC/MS/MS). In that capacity, the semi-quantitative nature of the ELISA response is often exploited to screen for samples where the target drug is present at concentrations above a defined cutoff, which is often greater than the formal limit of detection of the assay. While this is carried out to minimize the effects of cross-reactivity from endogenous matrix components, it is also exploited to minimize the number of confirmatory assays performed on samples with measurable drug concentrations that are not toxicologically relevant. We sought to exploit the semi-quantitative nature of the ELISA response to investigate the effects of tissue type and dose-death interval on the sensitivity (and therefore, probability) of detection of drug exposure through analysis of bone and marrow. This approach provides information on the effect of a particular variable on the likelihood of detection of drug exposure (e.g., tissue type or dose-death interval), although it does not indicate whether differences in sensitivity are due to differences in recovery of drug between matrices, or differences in drug concentration between different sample types.

The primary limitation of immunoassay methods in toxicological analysis is the potential for signal contributions from cross-reactivity of the immobilized antibodies with other components in the sample. Those components may be endogenous to the matrix, or they may be other drugs or toxins that happen to be present in the sample in question. In this work, we have expressed the ELISA absorbance data in terms of the relative change in absorbance of a given tissue extract measured relative to the absorbance values of matrix-matched extracts from the same tissue type. Our approach combined SPE for sample clean-up and ratiometric data analysis to correct, to some extent, for the presence of endogenous cross-reacting components, facilitating a more controlled comparison of response between different tissue types. The approach does not differentiate between signal contributions from the parent drug and those of cross-reacting metabolites. The data in Fig. 1 show that there is substantial cross-reactivity between diazepam, nordiazepam, and oxazepam. The ELISA kits used employ oxazepam as the enzyme-conjugated drug in the competitive binding reaction, and the manufacturer reports cross-reactivity to diazepam and nordiazepam in the ranges of 70–120% and 50–80%, respectively. Consequently, we refer to positive ELISA results as being indicative of diazepam exposure. Indeed, the presence of both parent drug and metabolites was confirmed in some cases by the confirmatory LC/MS/MS analysis (Fig. 3 and Table 1).

Both the binary classification sensitivity parameter and the %DA measurements, along with confirmatory LC/MS/MS measurements, should be considered in examinations of the sensitivity of detection of drug exposure under different conditions of tissue type and dose-death interval. Depending on the limit of detection of the analytical method used, and the sample preparation steps undertaken, there may be significant differences in the analytical response to samples corresponding to different experimental conditions, yet the drug(s) may be designated as *detected* in all cases. This is illustrated in comparison of the ELISA response to extracts of marrow with those of extracts of epiphyseal bone (Table 1). Furthermore, the mass of tissue sampled should also be considered. In this work, the mass of marrow sampled was significantly lower, by approximately one order of magnitude, than the mass of epiphyseal or diaphyseal bone from the same limb. The effect of this discrepancy may be understood if %DA values are normalized with respect to the mass of tissue assayed (i.e., %DA/m), whereupon it becomes immediately obvious that the sensitivity of drug detection in marrow is substantially higher than that in mineralized bone. The mean mass of marrow analyzed was *c*. 0.04 g, while the mean masses of epiphyseal and diaphyseal bone fragments were 0.44 and 0.28 g, respectively. It should be noted that this treatment is not

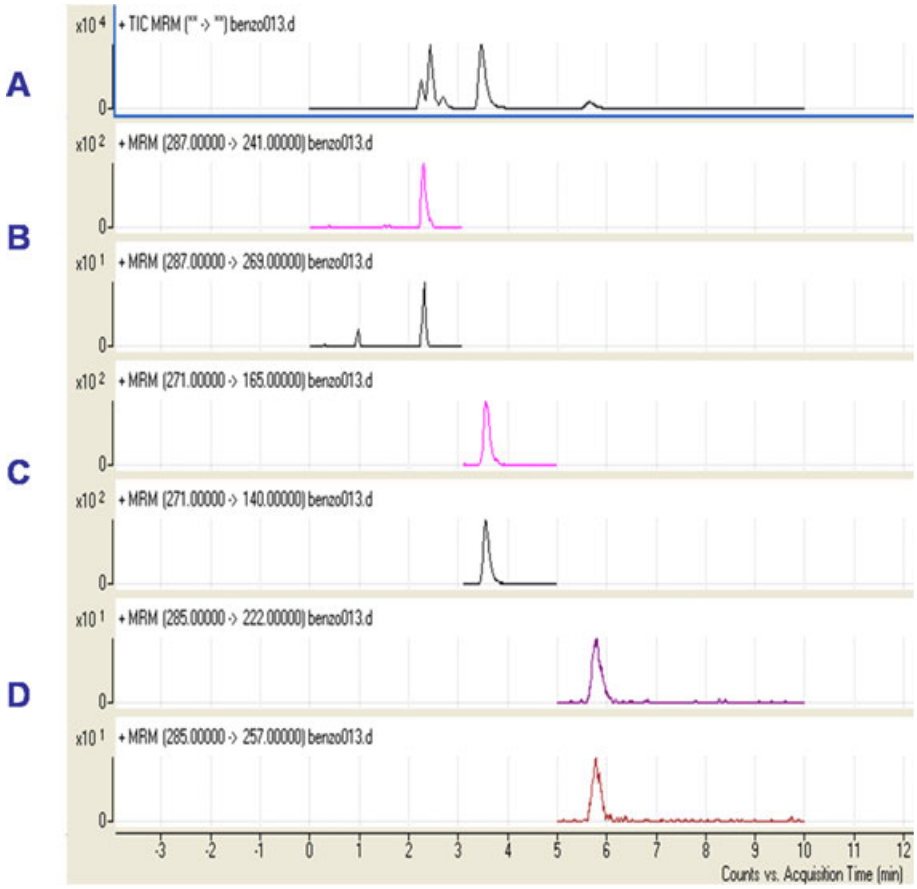

FIG. 3—*(A) Total ion chromatogram from LC/MS/MS analysis of diazepam positive marrow extract. (B) Multiple reaction monitoring (MRM) chromatograms of same marrow extract, corresponding to the transitions corresponding to oxazepam (287/241 and 287/269). (C) MRM chromatograms of same marrow extract, corresponding to the transitions corresponding to nordiazepam (271/165 and 271/140). (D) MRM chromatograms of same marrow extract, corresponding to the transitions corresponding to diazepam (285/222 and 285/257).*

strictly accurate in quantitative terms, as the relationship between %DA and drug concentration is nonlinear, but it provides a qualitative means of illustrating differences in the analytical value of different tissue types.

Comparison of %DA values within a given tissue type between different dose-death intervals showed that there was a trend toward decreasing sensitivity of detection as the dose-death interval increased. Interestingly, it was originally considered that the relatively small change in %DA values in marrow extracts at the 180 min dose-death interval (which corresponds to *c*. 3*t* in the rat) may have been due to high marrow benzodiazepine concentration, corresponding to the plateau region of the standard curve (Fig. 1). Reanalysis of those samples following a four-fold dilution yielded the same pattern of response with respect to dose-death interval, even though %DA values were less than 60% in all cases (pseudo-linear region, Fig. 1). Mean %DA values for the diluted samples were 44% ± 9%, 48% ± 6%, and 24% ± 7% for the 20, 60, and 180 min dose-death intervals, respectively. This pattern of response may be indicative of delayed or slow drug absorption, in comparison to that observed by Walker et al. (9), where maximal plasma diazepam concentrations were achieved within *c*. 10 min of intraperitoneal injection of 20 mg/kg. Alternatively, this pattern may be the result of relatively slow drug accumulation into the lipid rich marrow and then into the bone, followed by slower, gradual diffusion back into the circulating blood as elimination proceeds. In such a situation, the skeletal tissues may be considered to act as a "deep compartment" (10). In contrast, the work of Walker et al. (9) showed a rapid initial decline in plasma diazepam concentration, attributed to distribution to the peripheral compartment, followed by a slower elimination phase; the net pharmacokinetic profile described using a biexponential, two-compartment model. If the phenomenon observed in this work is due to slow diffusion of the drug from the marrow back into the blood as metabolism proceeds, then it becomes critical to assess the potential for drug detection at extended dose-death intervals, such as those longer than the time required for complete drug clearance from the blood, as this may impact the interpretation of a positive drug screen result in terms of the potential time window for drug administration. Full characterization of this effect will require further quantitative analysis examining the time course of benzodiazepine detection over longer extended dose-death intervals, and with corresponding blood samples, and will be the subject of future work in our laboratory.

These data may have significant implications for drug screening and analysis in human tissues. If the site-dependent ELISA sensitivity observed here, as has been observed in experiments involving acute ketamine administration in rats (7), is reflective of heterogeneous drug distribution in a given bone, then there is the possibility that partial bone sampling (as would likely be performed in analyses of human bone tissues) may yield false negative results. 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.

Finally, it is noteworthy to reiterate that while the use of experimental animals provides a means to control a number of experimental variables which cannot be controlled in forensic scenarios, absolute quantitative measurements generated in such research have limited direct applicability to analysis of human tissues. Consequently, the use of semi-quantitative immunoassay measurements is valuable in basic research. In addition to their high sensitivity, low sample volume requirements and high throughput, they may provide valuable information in terms of the effects of various experimental variables, including tissue type and dose-death interval on the sensitivity of drug detection in skeletal tissues.

Conclusions and Summary

These data have shown that ELISA may be a valuable tool in screening of skeletal tissue samples for drugs of abuse. These results are consistent with those presented in earlier work suggesting that marrow appears to be a significant depot for diazepam distribution and that epiphyseal bone fragments may provide a more reliable and sensitive sampling site than diaphyseal bone from the mid-femoral region. The data also suggests that the time delay between drug administration and death (the dose-death interval) may have a significant effect on the sensitivity of drug detection in skeletal tissues. Significant reductions in detection sensitivity were observed over relatively short dose-death intervals ($\sim$2–3*t*), although diazepam exposure was still detectable in 100% of marrow extracts and 60% of epiphyseal bone extracts after this time period. Further 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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References

1. Raikos N, Tsoukalia H, Njau SN. Determination of opiates in postmortem bone and bone marrow. Forensic Sci Int 2006;13:140–1.
2. Horak EL, Jenkins AJ. Postmortem tissue distribution of olanzapine and citalopram in a drug intoxication. J Forensic Sci 2005;50(3):679–81.
3. Kudo K, Sugie H, Syoui N, Kurihara K, Jitsufuchi N, Imamura T, et al. Detection of triazolam in skeletal remains buried for 4 years. Int J Legal Med 1997;10:281–3.
4. McIntyre I, King CV, Boratto M, Drummer O. Postmortem drug analyses in bone and bone marrow. Ther Drug Monit 2000;22:79–83.
5. Gorczynski LY, Melbye FJ. Detection of benzodiazepines in different tissues, including bone, using a quantitative ELISA assay. J Forensic Sci 2001;46(4):916–8.
6. Guillot E, de Mazancourt P, Durigon M, Alvarez JC. Morphine and 6-acetylmorphine concentrations in blood, brain, spinal cord, bone marrow and bone after lethal acute or chronic diacetylmorphine administration in mice. Forensic Sci Int 2007;166:139–47.
7. VandenBoer TC, Grummett SA, Watterson JH. 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. J Forensic Sci 2008; 53(6):1474–82.
8. Winek CL, Pluskota M, Wahba W. Plasma versus bone marrow flurazepam concentration in rabbits. Forensic Sci Int 1982;19:155–63.
9. Walker MC, Tong X, Brown S, Patsalos PN. Comparison of single-dose and repeated-dose pharmacokinetics of diazepam. Epilepsia 1998; 39(3):283–9.
10. Klotz U, Antonin KH, Bieck RR. Comparison of the pharmacokinetics of diazepam after single and subchronic doses. Eur J Clin Pharmacol 1976;10(2):121–6.

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