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Detection of Benzodiazepines in Different Tissues, Including Bone, Using a Quantitative ELISA Assay

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ABSTRACT: Benzodiazepines were analyzed in different tissue samples, including bone, by ELISA. The sensitivity of detection for different benzodiazepines was consistent with the manufacturer's reports of the cross reactivities of the antibodies used, with the greatest sensitivity for midazolam and the least for diazepam; in addition the pharmacokinetics was consistent with the known duration of action of the different benzodiazepines, with midazolam cleared rapidly, and diazepam slowly. Following intramuscular injection of 300 μg of midazolam at 16 h intervals for ten days, the drug was detectable in bone tissue samples obtained from skeletonized remains buried in soil at room temperature for three weeks.

KEYWORDS: forensic science, benzodiazepine, ELISA, bone

Traditionally, forensic toxicology involves the analysis of biological samples such as blood and urine (1). The search for methodologies that can extend these investigations to detection of different chemicals, and/or in different tissues, continues to drive one arm of research in this field (2). This becomes of particular pertinence in the field of criminal investigation, when often only skeletonized remains are available for investigative purposes. One class of drugs commonly encountered in forensics cases is the benzodiazepines (3). The studies described below document the detection of benzodiazepines in rodent tissue samples, including buried bone tissue from extensively decomposed/skeletonized bodies, using a quantitative ELISA technique.

Materials and Methods

Mice—Inbred 20 g C3H/HEJ mice, purchased from the Jackson Laboratory (Bar Harbor, ME), and 40 g Lewis (LEW) rats, purchased from Sprague-Dawley farms, were used.

Antibodies—Alkaline phosphatase conjugated rabbit anti-sheep IgG (A130-101AP); sheep anti-benzodiazepine (1100–5004); and mouse anti-benzodiazepine (MAB220), were purchased from Cedarlane Laboratories (Hornby, ON).

Benzodiazepines—Midazolam (1 mg/mL, 101236) and diazepam (5 mg/mL, 239809B) was provided by SRBEX; lorazepam (4 mg/mL, 101356) was obtained from Wyeth-Ayerst Canada Inc.

Serum and Tissue Samples—Serum was obtained at varying times post administration of drug from individual mice as described in the individual experiments. Brain, kidney, liver, lung, bone marrow, and bone tissue (after flushing to remove bone marrow) samples were also obtained, homogenized in 1 to 3 mL of phosphate buffered saline (PBS), centrifuged (500 g, 5 min, 4°C) and the clarified supernatants (6000 g for 10 min at 4°C) used for subsequent drug assays.

Administration of Benzodiazepines—Mice and rats received intravenous injection of various benzodiazepines in 300 μL PBS at the maximum tolerated acute dose; for midazolam, lorazepam, and diazepam these doses were 15, 20, and 20 mg/kg respectively. The maximum tolerated dose given by gavage was approximately 2 to 3-fold higher in all cases. Serum and tissue samples were collected at different times post administration and assayed for benzodiazepines by ELISA (4). In some studies, mice received chronic injection of 300 μg midazolam every 16 h for ten days before assay. In a final study, drug was analyzed in tissue from groups of mice receiving chronic administration of midazolam or PBS for 10 days prior to burial in autoclaved soil under sterile conditions, or in non-sterilized soil.

Sandwich ELISA for Specific Benzodiazepine Detection (Adopted from Current Protocols in Immunology, John Wiley Publisher, 1999)—ELISA plates (Falcon, Franklin Lakes, NJ, 353912) were coated with 50 μL of 1:200 dilution of monoclonal mouse antibenzodiazepine antibody. Wells were "blocked" with 200 μL of 10% Fetal Calf Serum (GIBCO, USA) and pulsed with 10 μg/mL of antigen for 4 h at room temperature. Optimal results were achieved using plates with bound midazolam, and subsequent results are described only for experiments using this drug. A 1:100 dilution of sheep antibenzodiazepine antibody was added, with test samples or known amounts of benzodiazepine (to construct a standard curve) as a competitive inhibitor for binding to plate-bound benzodiazepine. Plates were developed using alkaline-phosphatase coupled rabbit anti-sheep antibody and p-nitrophenyl phosphate substrate (Bio-Rad, Canada), measuring absorbance in an ELISA plate reader (Bio-Rad, Canada) at 405 nm. Control wells contained no sheep antibenzodiazepine antibody. All sample assays were performed in triplicate, and arithmetic means (±SD) calculated. In assays performed with samples obtained from buried tissue, where liquefaction of tissue had oc-

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curred, assays were performed using the protease inhibitor aprotinin (5 μg/mL).

Statistical Analysis—Individual data within groups were pooled (arithmetic mean ± SD). Equivalent groups (same tissue samples) within experiments were compared by pair-wise Student's t-test.

Results

Sensitivity of Detection of Different Benzodiazepines in Sandwich ELISA

Data in Fig. 1 show results from one of six studies to compare the detection thresholds for inhibition in ELISA by lorazepam, diazepam, and midazloam. Midazolam was the most potent inhibitor, followed by lorazepam, with diazepam showing 50% inhibition at high concentrations (10 μg/mL or greater). These data concur with the manufacturer's report on the antibody cross reactivity (Cedarlane Laboratories, Hornby, Ontario). Sensitivity of detection for midazolam was of the order of 50 to 100 ng/mL, with lorazepam detectable at 2-fold and diazepam at 10-fold higher concentrations.

Note that even when delivered by gavage in high dose (300 mg/kg) as a crushed "slurry," we found little absorption of powdered triazolam, lorazepam, or diazepam in rats or mice. In contrast, gavage or intravenous administration of soluble diazepam, lorazepam, or midazolam, produced rapid sedation at 20, 20, and 15 mg/kg, respectively. In all subsequent studies, drugs were administered by intravenous infusion. The clearance time ($t1/2$) from serum for midazolam/lorazepam following intravenous infusion of 300/400 μg was 25 and 48 min respectively (not shown). The dose to achieve sedation in mice (~20 mg/kg) was some 200 to 600-fold more than that needed in humans.

Detection of Benzodiazepines in Tissues

Benzodiazepines in different tissues were assayed following either acute challenge (300 μg midazolam, given 30 min before tissue harvest) or chronic challenge (300 μg midazolam given intramuscularly every 16 h for ten days). Control groups of mice received intravenous infusions of PBS alone prior to sacrifice. Data for both series of studies are shown in Fig. 2, with the drug (μg/tissue) shown for the *treated* mice only. No midazolam was detected in controls (data not shown).

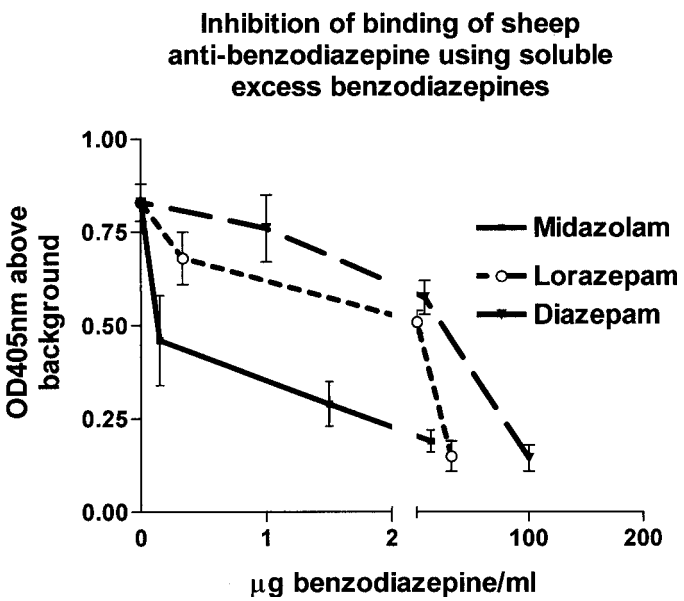

FIG. 1—*Competitive inhibition ELISA with plate-bound midazolam for detection of different benzodiazepines. Data show arithmetic mean (±SD) for triplicate determinations. Background OD readings (no plate bound drug) was 0.08 ± 0.015. The concentration of drug used for inhibition is shown on the abscissa.*

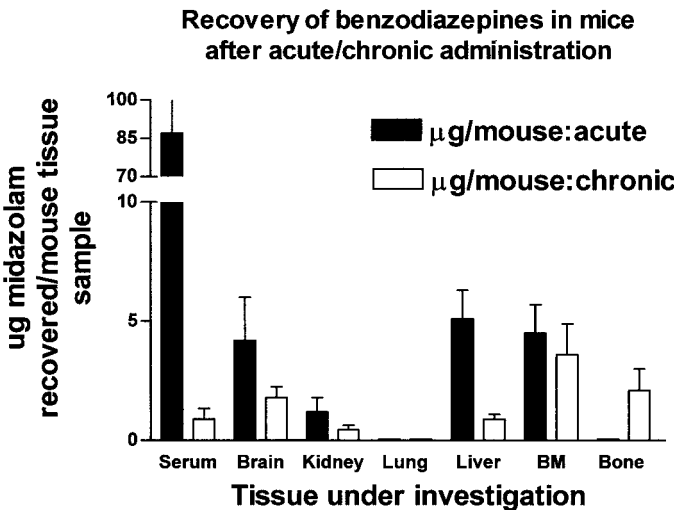

FIG. 2—*Midazolam recovered in different tissues following acute/chronic drug administration. Data (mean ±SD) are from three individual mice/tissue, each assayed in triplicate. Quantitation of drug used a standard curve as shown in Fig. 1. * indicates* $p < 0.05$ *for comparison of equivalent tissue groups. No significant drug was detected in tissues of normal, nondrug treated, mice (data not shown).*

After acute exposure, drug was detected in serum, liver, and bone marrow (both hematopoietic organs), as well as the brain (as anticipated from the sedative effect of the drug). Following chronic injection, but not after acute injection, drug was detectable in the bone tissues of treated mice. No independent analysis was performed to ensure that all bone marrow and other connective tissue had been removed from the bone tissue. Thus we did not formally exclude the possibility that the midazolam assayed was not in bone necessarily, but in tissues exogenous to bone.

Persistence of Immunologically Detectable Benzodiazepines in Different Tissues Postmortem

Groups of mice received chronic administration of midazolam as in Fig. 2, over ten days. Sixteen hours after the last injection serum was obtained, animals were sacrificed, soaked in detergent for skin cleansing, and stored as follows: frozen (−20°C); buried in sterilized soil; and buried in nonsterilized soil. Mice in both of the latter two groups were stored at room temperature (25°C). Twenty-one days later the bodies were retrieved, and different tissues extracted for ELISA (Fig. 3). Control groups, sacrificed at the time of the assay, included noninjected mice (negative control) and mice receiving chronic injections of midazolam, up to 16 h before sacrifice (positive control—Fig. 2).

These data confirm those of Fig. 2, and extend these observations significantly. Midazolam was still detected in animals three weeks postmortem, in bodies stored under conditions showing significant liquefaction and tissue degradation (room temperature, sterilized soil). A positive signal was seen even with bone tissue samples stored in nonsterilized soil, which showed much more degradation. Given the liquefaction and diminished tissue recovery

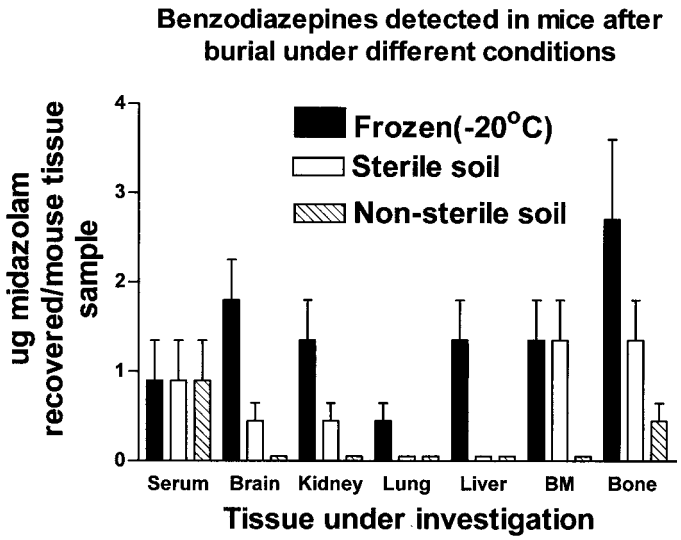

FIG. 3—*Tissue detection of midazolam in material stored under different conditions for 21 days. See text, Materials and Methods, and legend to Fig. 2 for more details. * indicates* $p < 0.05$ *for comparison with equivalent tissue harvested from material retrieved from sample buried in non-sterile soil.*

in this group, and the absence of midazolam in bone marrow (see Fig. 3), the midazolam detected in the bone tissue in this group most likely is in bone, and not in exogenous tissues. By extrapolation, we feel the same may be the case in Fig. 2.

Discussion

Benzodiazepines are frequently encountered in forensics cases (5). Our studies document the detection of benzodiazepines in rodent tissue samples, including bone marrow and bone tissue itself (Figs. 2, 3), using a "sandwich" ELISA technique (Fig. 1). While detection of benzodiazepines in bone marrow samples has been reported by other workers (6), this represents the first report of detection of the drug in bone tissue itself. While no independent analysis was performed to ensure that all bone marrow and other connective tissue had been removed from the bone tissue sample, we feel the data of Fig. 3 argues in favor of the hypothesis that the midazolam detected is indeed in the bone itself, and not in tissues exogenous to bone. The assay used is limited by the specificity and sensitivity of the antibodies used for detection of benzodiazepines. Techniques are potentially available to increase the sensitivity of the ELISA, including manipulating the concentrations of midazolam bound to the target ELISA plate; the sheep anti-benzodiazepine added as a detecting antibody; and even using biotinylated antibodies (and subsequent addition of streptavidin coupled developing reagents) to amplify the signal.

As determined by the serum half-life, the relative handling of different drugs in rodents is akin to humans, though the dose to achieve sedation is some 200 to 600-fold greater in rodents. Differences in the bioavailability and metabolism of this class of drugs in rodents and humans was evident from preliminary studies using gavage of the oral formulations of triazolam, lorazepam, or diazepam, at doses some 1000-fold greater than in the human population (Gorczynski, L.-unpublished data). During the development of this class of drugs by the pharmaceutical industry, cats were chosen (by Hoffman-Laroche, Basel), as an animal model system. Whichever animal model is used, however, there will inevitably be concern over direct extrapolation of the data to human studies.

Little is known about the postmortem changes of benzodiazepines and their metabolites. Data of Fig. 3 indicate that following brief (21 days) internment, immunologically detectable material is present, even in degraded bone tissue, from buried animals. The signal detected is a minimal estimate, because of nonspecific protease (and other) inhibitors in the liquefied tissue. Whether the "signal" is from unaltered drug, an immunologically active moiety of the degraded drug, or a signal resulting from a drug-dependent process (but not the drug itself) remains to be clarified. Investigations with gas chromatography would help resolve this issue. Nevertheless, our data are the first to show detection of benzodiazepines in bone tissues following chronic administration in rodents. Whether similar results are observed in humans, and whether other drugs (e.g., alcohol) confound these data, remains to be tested.

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