

Challenges in forensic toxicology of skeletonised human remains

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DOI: 10.1039/b609130j

Forensic toxicologists typically work with body fluids such as blood or urine, as well as visceral tissues such as liver. Very little work has been done to properly understand the utility of drug concentrations in bone tissue in a toxicologic examination. Literature reports suggest that detection of selected drugs in bone tissues is possible, but challenging work remains to determine the implications of bone tissue drug concentration measurements with respect to timelines of drug ingestion and deposition into bone, tissue sampling and sensitivity requirements, and environmental effects on measurements and their interpretation.

Some of the most notorious stories in the popular media focus on death investigations. A forensic toxicologist assists in these investigations through analyses of samples for the presence of drugs, alcohol, poisons and their metabolites in an effort to assist in the determination of a cause of death, impairment, exposure to a particular drug or poison, or compliance with prescribed medication. The types of samples that are typically encountered include blood, urine, liver, vitreous humour and stomach contents, although kidney, heart, brain and cerebrospinal fluid may also be examined. Whenever possible, blood is analysed, since the effects of a drug can be most accurately correlated with its blood concentration. Interpretation of drug concentrations with respect to toxic effects is more difficult with measurements using other tissues, as a result of variable distribution patterns between blood and the tissue in question (*e.g.*, urine or saliva); inhomogeneous distribution within a given tissue or a simple paucity of research correlating tissue concentrations with toxic effects.

Toxicological analysis is complicated in those cases where the remains of the decedent have undergone significant decompositional change. In these instances, analyses of blood and visceral tissue samples are complicated by chromatographic interferences arising from the complex spectrum of decomposition

by-products. In some cases, this may be addressed to some extent through analysis of alternative fluids such as vitreous humour or cerebrospinal fluid for determination of certain compounds such as ethanol or heroin metabolites.^{1,2} These fluids may be more protected from alteration due to microbial action in decomposition, and from contamination with other substances due to trauma. Analysis of such fluids may therefore serve to reduce the confounding effects of postmortem synthesis or extracompartmental contamination (*e.g.*, from unabsorbed drug in the stomach) that may falsely elevate drug concentrations. As putrefaction progresses, analysis of blood-laden fluids collected from the pleural cavity is often one of the few remaining options for the determination of the presence of drugs or poisons. While it has been suggested that such fluids may be useful in the interpretation of postmortem drug concentrations in cases of extreme putrefaction,³ interpretation of drug concentrations in fluid samples where blood may have been mixed with other non-circulating fluids may be problematic. Generally, as the postmortem interval increases, the extent to which the bodily fluids sampled may represent antemortem circulating blood diminishes as cells undergo lysis, tissues liquefy and fluids become mixed.

There is increasing interest in the study of drug distribution in other matrices such as hair or bone tissue, which may be better protected from the processes of decomposition. In cases of death investigation where the remains of a body have

undergone significant decomposition or scavenging, the analysis of such tissue types may provide the only source of toxicological information. Measurement and interpretation of drug concentrations in bone tissue will require the development of an understanding of the disposition of drugs in bone tissues by means of investigations both in human and animal models, since it is inherently difficult to design control into analyses of human postmortem tissues (such as strict control of dose history, concomitant drug use, health status, cause of death, and postmortem interval and environment prior to sampling). The simple detection of a drug in bone tissue may be indicative of exposure to that drug, but that measurement alone does not provide any information about doses consumed, dose pattern (*i.e.*, acute *vs.* chronic use) or restrictions on possible times of exposure. Understanding of the disposition of various drugs (and their metabolites) with different chemical characteristics within bone tissue may help to improve the information that can be gleaned from a measured bone drug concentration and ultimately yield valuable information to an investigation. Investigation of the disposition of drugs in bone tissue will require the controlled experimental conditions (dose, dose timeline, interval between last dose and death) that may be achieved with an animal model, but extrapolation of those results to a human model has limited validity. However, until such time as analytical methods evolve such that bone tissue may be sampled from living

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subjects receiving drugs under controlled conditions and subsequently be quantitatively analysed for drug concentrations as a function of time, this approach will have to suffice.

Drug analysis in human bone tissue

There is a paucity of research on the analysis for compounds of toxicological interest in human bone and bone marrow samples from skeletonised remains. The reports that have been published thus far have consisted largely of individual case reports, with little indication of consistency in the particular bone tissue sampled, or of the method of sample preparation, and are summarised in Table 1. Noguchi, *et al.*, reported the analysis of vertebral bone marrow following solvent extraction of basic, neutral and acidic compounds and detection of amitriptyline by GC/MS in a case of the uncovering of a significantly decomposed body from which blood samples could not be recovered.⁴ This is one of the earliest reports of forensic toxicological analysis of bone marrow. In this case the only medical history described was a retrospective search of local pharmacies for any prescriptions for this tricyclic antidepressant in the decedent's name.

In contrast, McIntyre *et al.*, reported the quantitative analysis of a number of psychotropic drugs in both human femoral bone and bone marrow from a series of postmortem investigations. Drugs encountered included tricyclic and non-tricyclic antidepressants (*e.g.*, doxepin, mocoblemide and sertraline), antipsychotics (chlorpromazine, thioridazine and clozapine) and benzodiazepines (diazepam, oxazepam and temazepam); some metabolic products of these drugs were also detected.⁵ This study was somewhat limited in scope as cases where significant decomposition occurred were rejected. Bone samples were obtained from the mid-femoral region and were roughly 4 cm in length. Bone samples were prepared and underwent methanolic extraction as outlined in Table 1, prior to analysis by GC/MS or HPLC. Calibration of quantitative analysis of extracts involved equilibration of similarly prepared bone sections derived from bovine hindquarter in

Table 1 Summary of literature reporting bone analysis in human skeletal tissues

Author	Tissue sampled	Anatomical site of sampling	Tissue preparation	Extraction method	Drugs detected
Noguchi <i>et al.</i> ⁴	Marrow	Vertebrae	Homogenised and diluted in ethanol; ethanol filtered and evaporated; residue reconstituted in distilled water	Solvent extraction of aqueous solution for acidic and basic drugs; analysis of basic drugs by GC, GC/MS	Amitriptyline
Kudo, <i>et al.</i> ¹⁴	Marrow	Femoral segments	Digested with 2 M NaOH solution	Solvent extraction of digestion solution; analysis was targeted for triazolam by GC/MS	Triazolam
McIntyre <i>et al.</i> ⁵	Bone; marrow	Femoral segments	Bone was cleaned and cut into rings 1–2 mm in thickness; marrow was diluted in distilled water and homogenised	Bone was incubated in methanol; methanolic extracts underwent further solvent extraction;	Doxepin, Mocoblemide, Sertraline, Chlorpromazine, Thioridazine, Clozapine, Diazepam, Oxazepam, Temazepam
Horak and Jenkins ⁷	Bone	Iliac segments	Bone was cleaned and fragmented with surgical scissors	Bone was incubated in methanol; methanolic extracts underwent further solvent extraction for basic drugs	Citalopram
Raikos <i>et al.</i> ¹⁵	Bone; marrow	Femoral segments	Bone ground in mortar, demineralized with 3 N HNO ₃ ; marrow was diluted in distilled water and homogenised	Solvent extraction of digestion, homogenate solutions for basic drugs	Morphine

methanolic drug cocktails over the range 0.05–0.5 mg kg⁻¹. The authors noted assay detection limits in the order of 0.01–0.02 mg kg⁻¹ for most analytes, with good linearity. The authors also indicated that preliminary investigations revealed that <10% of residual drug was extractable from further methanolic incubation subsequent to the first extraction cycle, suggesting that the efficiency of methanolic incubation was relatively high. Calibration may be problematic with this method, as authors attempted an equilibrium solvent extraction of drugs from relatively large solid fragments of bone. Calibration of the quantitative response involved equilibration of standard drug-free bone fragments obtained from another species with methanolic solutions of the relevant drug, a treatment that cannot account for differences in extraction efficiency between samples.

In some cases, both parent drug and metabolite were detected, but quantitative ratios of parent drug : metabolite concentrations differed between bone and blood samples. This may be reflective of different distribution patterns of drugs between the two tissue types, as has been observed with drug analysis in hair.⁶ The drugs in question were detected in 25 of the 29 cases where positive blood concentrations were quantitatively measured, implying that in certain cases analysis of bone tissue using this method may not yield any toxicologically relevant information. In 3 cases, temazepam and chlorpromazine were detected in bone tissue but could not be confirmed in blood.

The same study reported the analysis of bone marrow for the presence of drugs. In one part of the study, marrow derived from the bone study was stored frozen (–20 °C, unpreserved) for a period of roughly 3 years before undergoing analysis for the drugs described above. Bone marrow was diluted with deionised water, and homogenised prior to undergoing extraction by standard procedures for analysis by HPLC and GC/MS. These analyses resulted in detection of drug in only 45% of cases where they were detected in corresponding blood samples. In a more controlled study where marrow was extracted from lumbar bodies, diluted in deionised water and subjected to standard drug

extraction protocols without lengthy storage, drugs were detected in marrow in 70% of the cases where they were detected in the corresponding blood samples. Clearly, instability of drugs in marrow or marrow extracts, as well as blood–bone and blood–bone marrow distribution ratios are important experimental variables that require elucidation for all drugs of interest.

A study of a single fatality of a psychiatric patient within a group home for mentally ill adults was reported by Horak and Jenkins.⁷ The cause of death was reported as acute intoxication by a combination of citalopram and olanzapine. In this case the authors analysed a number of tissues in an attempt to elucidate the postmortem tissue distribution of these drugs. Iliac bone tissue was prepared by incubating 1 g bone tissue, which had been cut into slivers using surgical scissors, in distilled water or methanol for 24 h. Aliquots of these extracts then underwent standard drug extraction protocols used for the isolation of drugs from blood samples. Analysis was done by GC-NPD with qualitative confirmation by GC/MS. Qualitative (*i.e.*, “positive” or “not detected”) results of bone analysis were reported only, however the authors indicated that the methanolic extraction procedures were significantly more efficient than aqueous extraction. In this case, citalopram, but not olanzapine, was detected in bone tissue. This case was significant since it represented a scenario where the measured postmortem blood drug concentrations were highly elevated and amongst the highest observed by the authors in their laboratory, yet olanzapine was not measurable in the post-mortem bone tissue samples. It is important to note that olanzapine has been observed in previous research to break down in blood samples *in vitro*.⁸ Further information with respect to the dosing history of the decedent may be helpful, but a fundamental understanding of the stability and disposition of these drugs in bone tissue is lacking.

The cases described thus far typify the problem routinely encountered in forensic toxicology, where the case histories reported provide few details as to the pattern of drug use. The reports of McIntyre *et al.*,⁵ and Horak and Jenkins⁷ suggest that not all drugs may be

detectable in bone or marrow samples in all cases where they are measurable in blood. This is a significant problem in those cases where there are no other tissue samples available for analysis, such as the report by Noguchi.⁴ It will be critically important to understand drug instability in bone marrow and to optimize methods in terms of sensitivity and accuracy to fully understand the distribution of drugs in these tissues.

Preparation of bone samples for analysis requires an extraction step that generally makes use of an organic solvent (*e.g.*, methanol) or demineralization with strong acid treatment. In the cases described here, extraction was done by incubation with methanol. While it may be possible to homogenise a marrow sample with relative ease, the heterogeneous nature of solid bone requires that significant processing of the sample (*e.g.*, grinding) be done to facilitate maximal surface area for contact with extraction solvents. Where bone is processed into relatively large fragments, incomplete diffusion of the solvent into the matrix is likely, resulting in incomplete and inconsistent solvation and extraction of drugs from within the matrix. This limits the utility of an internal standard in correcting for matrix effects and limits the accuracy of calibration based on incubation of blank bone fragments in standard drug solutions prepared in a solvent such as methanol.

Toward an understanding of the disposition of drugs in bone tissues

The disposition of drugs in bone tissues has been examined in a preliminary fashion in animal studies reported by Winek *et al.*^{9–11} as well as by Gorczynski and Melbye.¹² The studies of Winek *et al.* have shown that a number of drugs (desipramine, flurazepam, ethanol, methanol, isopropanol, pentobarbital) may be detected in rabbit bone marrow shortly after death and that, under the relatively high and limited dose conditions reported, show some correlation to corresponding plasma drug concentrations. These studies also introduce data that suggest that some marrow drug concentrations may decrease over time, and that in the case of ethanol the temporal behaviour of marrow ethanol

concentration may follow a more complex pattern including both increases and decreases, depending on environmental temperature. A study reported by Gorczynski and Melbye described the immunochemical detection (ELISA) of the benzodiazepines midazolam, lorazepam and diazepam in both bone and bone marrow of mice and rats following both acute and chronic dosing, and following various conditions of storage in the postmortem period (*i.e.*, frozen, as well as buried in both sterilized and non-sterilized soil).¹² This study was the first to report the detection of these drugs within bone itself as opposed to in bone marrow. It also showed differences in assay responses following acute *versus* chronic dosing, as well as in animals exposed to different environments in the postmortem period. This study, and those presented by Winek *et al.*, represent an important starting point in answering fundamental questions with respect to the disposition of drugs in bone tissues. Building on this approach with study by quantitative methods that can identify and resolve drugs from metabolites or other degradation products may help to elucidate important information about the measurement and interpretation of drug concentrations in bone and bone marrow.

Physiological considerations

Some of the information critical to understanding the distribution of drugs within skeletal tissues follows from the structural and physiological features of the various bones. Different bones within the skeleton have different architectures

and relative proportions of different types of bone tissue. Bone is a non-uniform tissue, comprised of two main types: compact or cortical bone (a dense, solid matrix) and cancellous (spongy) bone, which may be described as being somewhat porous, with open space that often contains marrow, bound by projections of needle-like trabeculae. An example of segments of a human femur illustrating these architectural differences is shown in Fig. 1. These architectural differences imply differences in surface area over which the mineralized bone matrix is in contact with cells responsible for bone formation and resorption (osteoblasts and osteoclasts),¹³ which may in turn serve as storage sites for drugs. In long bones the cancellous bone tends to be more concentrated near the ends, or epiphyses, of the bone, while compact bone dominates along the length, or diaphysis, of the bone. Both compact and cancellous bones are well vascularized. Long bones also have a thick membranous sheath (*periosteum*) covering most of their surface area. This membrane serves more than a protective or structural role and is rich in bone forming and bone destroying cells, as well as vasculature to ensure that those cells are fed. Long bones have a cavity within the diaphysis called the medullary cavity; the medullary cavity is typically filled with yellow marrow in adults, which is a connective tissue that is rich in lipid content. Overall then it may be imagined that drug distribution may vary spatially within a bone and according to bone type, depending on the relative lipophilicity of the drug and the

composition of the bone in question. Drug distribution may also vary as a function of the age and health status of the subject, with factors such as disease state (*e.g.*, osteoporosis, anaemia) playing potentially significant roles. Furthermore, the processes of decomposition are very likely delayed within bone tissue and thus temporal changes in drug concentration are unlikely to parallel those observed in blood.

Forensic considerations

In order for drug analysis in skeletal tissues to find use in forensic investigation, the forensic implications of analytical results must be clarified. Work in both human and animal tissues to date suggests that there may be some difficulty in correlating postmortem drug concentrations in blood with those in bone or marrow, implying that non-detection of drugs in these tissues may be possible in cases where drugs may have played a significant role. This suggests that a better understanding of drug distribution and fate within these tissues is required. Furthermore, correlation of bone drug concentrations with toxic effects cannot properly occur without controlled analysis in living subjects; a task that does not seem possible given the ethical considerations and current technical capabilities.

Overall, a number of questions need to be answered, that require sensitive and reproducible analysis of drug concentration in bone tissues. The development of extraction methods that can provide information about extraction efficiency and precision of drug extraction from both solid bone and bone marrow are required. The concentrations at which various drugs (with different lipophilicities and acid–base properties) may be detectable in bone tissue must be surveyed and documented. While this may be possible in controlled experiments with animal models through use of radiolabeled drugs, optimization of sensitivity in analytical methods that may be applied to real forensic samples (*e.g.*, LC/MS) is required for this methodology to find routine use in casework. How much, if any, spatial variation is there in bone drug concentration? Given that different bones have different structural features, and relative amounts

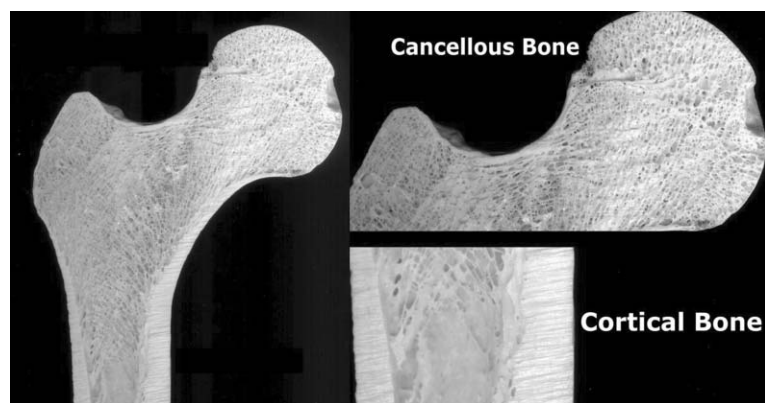


Fig. 1 Proximal segment of human femur. Expanded segments show cancellous bone (upper) and cortical bone (lower). Sample provided courtesy of Dr S. Fairgrieve, Dept. of Forensic Science, Laurentian University.

of compact and cancellous bone, as well as different relative amounts of red and yellow marrow, then how much, if any, variation is there in bone drug concentration between different bones? Answers to these questions will facilitate the establishment of optimum sampling sites for the most sensitive detection of various drugs of interest.

Once the sampling site(s) associated with the most sensitive detection of a particular drug is established, the time-course of drug concentration in bone tissue following both acute and chronic use at various dose levels may be more completely addressed. Further important information that may be then collected is the relationship between bone and blood drug concentrations under various conditions of drug use, and how long after death a particular drug can be detected in bone. This latter question is dependent in turn upon how environmental conditions affect drug detection in bone tissue. If there is significant spatial variation in bone drug concentration, it may be significantly more challenging to determine if a drug is stable within the bone matrix under a given set of environmental conditions. It has been clearly established over years of research that certain drugs are susceptible to degradation *via* simple hydrolysis (*e.g.*, cocaine) or bacterial action on certain functional groups (*e.g.*, the reduction of the nitro functional group in the

nitrobenzodiazepines—nitrazepam, clonazepam and flunitrazepam). The issue of postmortem redistribution should be addressed with respect to whether bone drug concentrations are susceptible to temporal changes in the same manner as those observed in blood derived from central compartments such as the heart, where drugs diffuse along a concentration gradient from one site to another, such as from within a particular tissue to blood nearby.

It is reasonable that in order to achieve control in such characterization experiments, animal models are the most appropriate choice. In doing so then, extrapolation of results to human models is speculative in nature, but this work may provide mechanistic information that will guide sampling and analytical method choices in work with human tissues. Subsequent drug concentration measurements in human bone may then be made with the promise of greater information content with respect to time-lines of drug ingestion and the influence of environmental factors. It is important to remember that correlations between drug concentration in bone marrow and pharmacological effect may not be clear, so that interpretation or estimation of toxicity from such analyses may be of limited value. Once the disposition of a drug within human bone tissue has been characterized, the scope of forensic information that may be gleaned from a

bone drug concentration measurement will be better defined.

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