Molecular and Integrative Toxicology

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Bone Toxicology

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Bone Toxicology

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Foreword

Toxicology is often defined as the study of the adverse effects of chemical, physical, or biological agents on organisms and ecosystems. Toxicologists employ a variety of in silico, in vitro, and in vivo experimental approaches to aid in the characterization of these effects. Within the discipline of nonclinical safety assessment of drugs, biotherapeutics, and environmental chemicals, in vivo toxicology studies are used to survey a broad range of organs and tissues to determine if there are any effects on the structure and function of an organ system. While the range of systems examined in these studies is quite broad, much of the focus has been on what are often referred to as major organ systems and there are discrete subdisciplines (e.g., hepatotoxicity, neurotoxicity, nephrotoxicity) which focus on a specific organ system. These subdisciplines have a robust literature spanning many decades and often have multiple comprehensive textbooks and journals focused on a single organ or system. There are a variety of other organ systems for which there is a rich literature; however, there are no comprehensive review textbooks available. The skeletal system is certainly one of the systems that has lacked from an expert review, and this volume entitled *Bone Toxicology*, edited by Susan Y. Smith, Rana Samadfam, and Aurore Varela, provides the first authoritative text in this area.

In the training programs of most toxicologists, there is limited or perhaps no training specific to bone biology or toxicology. Most toxicologic pathologists will have a base of knowledge in skeletal anatomy, physiology, and pathology from their training; however, they are typically not trained in some of the quantitative techniques which can be crucial to the study of the skeleton. As such, the casual student often perceives the skeleton, like other calcified tissues, to be a relatively inert and static organ system. This perception is often reinforced by the fact that practicing toxicologists and pathologists may have never or only rarely encountered the bone as a target organ of toxicity in their experiments. As readily demonstrated in this text, bone is a highly dynamic tissue which can be influenced by a variety of factors including both direct effects of a potential therapeutic or indirect factors such effects on body weight, food consumption, or other experimental factors. As with other systems, the bone does not work in isolation and has important connections and inter-relationships with a variety of systems, most notably the endocrine, neural,

and immune networks. The complex and dynamic nature of bone, combined with the increasing diverse range of targets and mechanisms of action of both small molecule and biologic therapeutics, has made it increasingly important for practicing toxicologists to have resources to better understand bone biology, have a working knowledge of appropriate endpoints to assess the skeleton, and appreciate how alterations in a variety of organ systems can impact the skeleton. This volume of *Bone Toxicology* provides such a comprehensive resource for the toxicology community.

It is important to remember that the role of nonclinical safety assessment is not just to identify the effects of potential therapeutic, chemical, or environmental agents on the test systems we study (i.e., hazard identification), but ultimately to understand what the relevance of those findings are for the conditions under which humans will be exposed to the therapeutic agent, chemical agent, or environmental agent (i.e., risk assessment). For disease states such as osteoporosis, much is known about the relative strengths and limitations of the various animal models for predicting responses in humans; however, for the types of effects encountered in repeat-dose toxicity models, the relevance to humans is often a very challenging question and there is often not an existing literature to draw on. By drawing on the information provided in this volume, the toxicology community will be better equipped to accomplish the goals of both detecting effects on the skeleton in animal models and assessing potential human risk. By accomplishing these goals, we will be better able to define reasonably safe conditions of use in the patients and consumers we serve.

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Preface

The skeleton is traditionally considered a hard structure providing support to allow locomotion, to protect sensitive internal organs, and to serve as a major reservoir for the maintenance of serum calcium. However, bone is a dynamic structure, recognized as having a pivotal role as an endocrine organ, and is coming into focus as an important target tissue in the overall development of a new drug, whether bone is the intended target or not. The skeleton is intimately related to other organ systems through paracrine, endocrine, and neural networks. The objective of this book is to provide the toxicologist in preclinical drug development with the necessary tools to identify and characterize a skeletal effect and to present current research on skeletal regulation and its role as an endocrine organ. This book is not intended to list bone toxic agents or detail their effects, unless needed to illustrate a point. The toxicity of many agents to bone is well described in the literature and is beyond the scope of this book.

The book is divided into three parts. Chapters 1, 2, 3, and 4 in Part I of the book introduce the overarching aspects and goals of skeletal evaluations in drug testing, as well as bone biology, regulatory aspects, pediatric applications, and important animal models. A basic knowledge of bone biology is fundamental for an appropriate assessment of effects of a drug treatment on the skeleton. Many lessons can be learned from Chap. 2, “Bone Physiology and Biology,” not the least of which is that a growing skeleton is very different from a mature, adult skeleton. Hence, Chap. 3 is dedicated to specific considerations for bone evaluations for pediatric therapeutics. We have learned much of our current understanding of bone biology from the testing of drugs intended for the prevention or treatment of osteoporosis; the use of simple animal models of osteopenia to test the efficacy and pharmacology of various drug classes has been fundamental to the successful approval of current osteoporosis drugs. These models, highlighted in Chap. 4, can be used to add important key data to a drug development program for many other indications.

In Part II, Chaps. 5, 6, 7, and 8 describe the methods used to derive the four primary outcome measures used to evaluate the skeleton: biochemical markers of bone turnover, imaging, histopathology and histomorphometry, and biomechanical strength testing. Each of these end points has been used extensively in bone research

for over 20 years. The adaptation of these end points for use in more general safety assessments of the skeleton has led to interesting challenges while broadening their application to encompass numerous species and important investigations of the juvenile skeleton.

Highlighting the importance of a systems biology approach to drug safety testing, the message that appears throughout the chapters is that bone is not an isolated tissue and is considered an endocrine organ with strong evidence accumulating to support cross talk with many other organ systems. Chapters 9, 10, 11, 12, 13, 14, and 15 in Part III of the book are devoted to topics on bone regulation, including interactions with muscle, pituitary hormones, the kidney, the immune system, the central nervous system, intestinal microbiota, and energy metabolism. These chapters were selected as "hot topics" because of important research advances in these areas and the development of new therapeutic targets; it is by no means intended to cover all possible known interactions of bone.

This book is intended to provide the toxicologist in nonclinical drug development with information on skeletal biology, regulatory requirements, and application of the tools, as well as an appreciation for the regulation of bone and its cross talk with other major organ systems, emphasizing the importance of a systems biology and weight of evidence approach to safety assessments.

Montreal, QC, Canada Susan Y. Smith

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Part I
Bone Toxicology in Preclinical Drug Testing

Chapter 1
Introduction and Considerations in Bone Toxicology

Susan Y. Smith, Nancy Doyle, and Melanie Felx

Abstract This chapter is intended to provide the researcher with information to facilitate in the design, execution, and interpretation of studies with bone end-points. The ability to use our knowledge of bone biology to ensure study conditions are optimal to detect an effect will be presented. This includes study design considerations and the utility of other models to explore the impact new compounds may have on the skeleton in the development of safe and effective drugs.

Keywords Regulatory • Toxicology • Safety • Bone end-points • Markers • Radiography • Bone densitometry • Histomorphometry • Biomechanics • Study design • Bone quality • Adversity

1.1 Introduction

Bone end-points can be included in any safety study design, for any species, at any age, irrespective of duration and route of administration. Additional mechanistic studies can also be designed on a case-by-case basis to meet regulatory demands. The ability to use our knowledge of bone biology to ensure study conditions are optimal to detect an effect is key to a successful outcome. This includes study design considerations, the utility of animal models (chemically, genetically, or surgically modified) in a safety assessment setting, and the potential benefit of including in vitro studies and studies using nonmammalian species (such as zebra fish). These studies cannot only provide invaluable mechanistic data but also respect the three R's initiative and the ethical use of animals.

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This chapter will also tackle the issue of skeletal adversity. One of the most difficult aspects of interpreting the relevance of a skeletal effect is to understand what constitutes a direct effect on bone and what might be considered secondary to effects on growth and body weight. This is a considerable challenge in therapeutic areas such as diabetes and obesity, for example, where the intended pharmacological outcome is likely to impact body weight in non-diseased animals. Animal health and welfare is always the primary concern and of relevance in studies which may become confounded by exaggerated pharmacological effects. Therefore, strategies for mitigating effects of exaggerated pharmacology are important considerations in study design.

The term "bone quality" has many definitions and is often used as a "black box" when unknown factors affect bone strength, which is a product of both bone quantity and bone quality. Bone quality encompasses all the geometric and material factors that contribute to fracture resistance (Donnelly 2011). Endpoints used to assess skeletal integrity therefore need to characterize the bone geometry and microarchitecture, assess the mechanical properties, and evaluate its tissue composition. No single method can address all aspects of bone quality, but a combination of techniques can help to provide a comprehensive evaluation. The toolbox available in a preclinical setting can be extensive; therefore, the methods used to assess bone should be tailored to the study design and the outcome measures of interest.

1.2 Regulatory Considerations

The primary outcome measures used preclinically to identify and characterize the bone quality are biochemical markers, radiography/bone densitometry, histopathology/histomorphometry, and biomechanics. The selection of these end-points has largely been driven by the requirements or guidelines issued by the regulatory agencies to test drugs intended to treat or prevent osteoporosis (osteoporosis testing guidelines: Japanese MHLW 1999; EMA 2007; FDA 2016b). These various techniques have been employed in bone research for over 20 years and most are successfully validated for use in a good laboratory practice (GLP) setting. GLP requirements may be important if data is intended to support a regulatory submission, but may not be required in early phase exploratory studies. Currently for toxicology studies, regulatory guidelines have been issued to assess skeletal growth only for therapeutics intended for pediatric use (pediatric guidelines: FDA 2006; EMA 2008; Japanese MHLW 2012), with requirements to study growth and skeletal development in detail in the pediatric investigational plans (PIP – EMA 2006) and pediatric study plans (PSP – FDA 2016a). In all other cases, a standard toxicology package typically includes the routine qualitative histopathological evaluation of hematoxylin and eosin-stained decalcified bone sampled from the sternum or femoral-tibial joint for large animal species and the distal femur, femoral-tibial

joint, and/or proximal tibia for rodents. Standard histopathology uses transmitted light microscopy and is an important diagnostic tool but is likely the least sensitive method to discriminate effects on bone mass or density; polarized light is also used to verify the lamellar organization of the bone matrix and to examine whether woven bone is present. If changes are seen, then they are probably important (major). This would be a trigger to use more sensitive techniques to discern an effect when looking at lower dose levels, to obtain information on the chronology of the change, and to further characterize the change. These specialized techniques include in vivo biomarkers such as biochemical markers of bone turnover or skeletal imaging, or ex vivo special histopathology stains (such as von Kossa or Goldner's trichrome) and/or histomorphometry on undecalcified tissue (see Chap. 8), and biomechanical strength testing (see Chap. 7). Skeletal imaging in most toxicology studies involves the use of radiography and/or bone densitometry. Bone densitometry equipment used extensively in preclinical research is dual energy X-ray absorptiometry (DXA), a 2-dimensional areal assessment with area, bone mineral content (BMC), and bone mineral density (BMD) as the primary output measures. Bone densitometry is also performed using peripheral quantitative computed tomography (pQCT) which provides three-dimensional measures of area, BMC and BMD, as well as separate measures of area, BMC and BMD for the trabecular and cortical bone compartments. Peripheral QCT scan data provides measures of several important geometric parameters: bone diameter (periosteal circumference), endosteal circumference, and cortical thickness. Software algorithms use these data to derive surrogate indices of bone strength including cross-sectional moments of inertia (CSMI). The advantages and disadvantages of these different skeletal imaging techniques will be further discussed later in this chapter and in Chap. 6 (Skeletal Imaging).

To date, qualitative histopathology remains the gold standard for diagnostic pathology and hazard identification in the safety assessment of potential new therapeutics, but it is not sensitive to evaluate changes in bone mass, geometry, and density. For diagnostic terminology used to describe bone changes, the reader is referred to the INHAND initiative publication devoted to skeletal tissues and teeth of laboratory rats and mice (Fossey et al. 2016). In most cases, qualitative histopathology has sufficient sensitivity to detect test article-related effects on bone marrow and growth plates in standard toxicity studies, although it may lack the sensitivity to detect effects of test articles on key physiological processes in bone tissue such as bone formation, mineralization, and resorption. The known limitations of standard qualitative histopathology and our current knowledge of bone signaling pathways related to specific drug classes has led to increasing demands from the regulatory authorities to include specialized end-points in toxicity studies to identify if the skeleton is a potential target tissue and/or to characterize a skeletal effect. Whenever the skeleton is considered potentially at risk, general toxicology studies should make provision to include retention of additional bones at termination to allow a more comprehensive skeletal evaluation, as needed, Table 1.1.

Table 1.1 Recommendations for bone retention in toxicology studies[a]

Bone	Potential use	Storage
Rodent/non-rodent		
Tibia, proximal	Histomorphometry/micro-CT	NBF then alcohol
Lumbar vertebrae L2 (NHP), L3	Histomorphometry	NBF then alcohol
Lumbar vertebrae L4, L5[b]	Biomechanics	Frozen −20 °C
Femur, whole	Biomechanics	Frozen −20 °C

NBF 10% neutral buffered formalin, *NHP* nonhuman primate
[a] Minimum recommendations in addition to standard regulatory requirements; additional backup bones can be added
[b] L4 + L5 (together) for mice

1.3 Tier Approach to Including Specialized Bone End-Points in Preclinical Toxicology Studies

The decision to include specialized bone end-points into toxicity studies is driven by numerous factors. These specialized techniques when used as part of a systems approach to safety assessments are normally sufficiently sensitive to detect perturbations in bone which are needed to address liability, to monitor drug pharmacology, determine the chronology of an effect, address a regulatory requirement, or provide mechanistic data to characterize an effect. In instances where markers show changes consistent with the expected pharmacology of a compound, blood sampling for pharmacokinetics can be combined with sampling for biochemical markers to establish a pharmacokinetic/pharmacodynamic (PK/PD) profile (e.g., see Ominsky et al. 2010). This PK/PD profiling can help guide selection of a specific marker for use in future studies and potentially to establish the optimal timing for sample collection.

Safety assessments ultimately need to provide information regarding how a drug is affecting the skeleton and whether this is a beneficial or adverse effect. When testing a compound belonging to a specific drug class with known class effects, these end-points can be used to confirm the class effect and to further characterize the drug for equivalence, superiority, or adversity. When testing a new drug with no established toxicity, based on what is known of the target or mechanism of action, standard clinical pathology parameters from acute and/or early dose-range finding studies can be monitored for perturbations in calcium/phosphorus and total alkaline phosphatase levels, as a minimum. Biochemical markers can be added to the clinical pathology analysis to facilitate hazard identification. These early data are used to drive the design of the sub-chronic and chronic studies. In studies 28 days or longer, radiography and/or bone densitometry can be added, either in vivo or ex vivo using excised bones. Radiographs are used to monitor skeletal growth (most notably in juvenile toxicology studies), assess skeletal maturity (growth plate closure), identify and/or monitor the progression of abnormalities and bone healing, and as a tool in skeletal phenotyping. Radiography has become an important end-point in rodent

lifetime pharmacology or carcinogenicity studies where there is a risk for osteosarcoma; whole body radiographs obtained prior to termination are used to identify bone masses and lesions to facilitate tissue collection at necropsy and subsequent microscopic evaluation and diagnosis (Jolette et al. 2006; Chouinard et al. 2016). In vivo bone densitometry is highly recommended for non-rodent species where group sizes are small (typically 3–5/sex) and bone density can vary considerably between animals. Inclusion of baseline scans for large animals allows any changes to be determined relative to individual animal baseline data. Baseline scans are normally not required for rodent studies which typically are well powered ($n = 8$ to 10/sex) and have relatively homogeneous bone density within a population. For rodent studies, adequate data for interpretation can often be obtained ex vivo. For rodent and non-rodent studies 3 months or longer, in vivo scans can be acquired at several timepoints during the study to provide a chronology of any effects.

To further characterize a skeletal effect, provision can be made in the protocol to retain appropriate bone specimens to perform additional end-points such as histomorphometry and biomechanical strength testing. Histomorphometry provides unique information on bone microstructure and the dynamics of bone turnover. Biomechanical strength testing is considered the ultimate test of bone quality and is normally performed on a long bone such as the femur and vertebrae. In humans, most osteoporotic fractures occur at the spine or hip; therefore, these are key sites to measure bone strength in animal studies.

High-resolution scanning using micro-CT imaging is currently not used routinely in a toxicology setting; however, application of this technology is expanding, particularly in developmental toxicology studies (Johnson et al. 2014). High-resolution images of whole fetuses can be rapidly assessed qualitatively for abnormalities. Current use of this technology is being standardized with respect to equipment, methodology, and terminology. Submission of image files for regulatory review is intended to expedite this aspect of the drug development process and allow the reviewer to verify reported abnormalities directly. Quantitative micro-CT, used to measure bone microarchitecture, provides a high-resolution scan comparable to histomorphometry structural parameters but without the lengthy processing time (plastic embedding, sectioning, and staining). Micro-CT provides a three-dimensional (3-D) evaluation and includes parameters such as tissue mineral density and connectivity index that cannot be derived using histomorphometry (Kazakia et al. 2008; Donnelly 2011). In toxicology studies, tools with a lower resolution (DXA, pQCT) that are less expensive to acquire and operate are normally adequate to detect changes in bone mass (which reflect changes in bone architecture). An important application of high-resolution CT scanning in bone quality studies is the ability to reconstruct the 3-D images to perform finite element analyses (FEA) to model bone strength (Keaveny 2010). Data from animal models are then compared with actual biomechanical strength data. The micro-CT data from animal studies are compared with clinically acquired data and used as a translational tool to identify effects on bone strength without the need to perform biomechanical testing in humans. Baseline (pre-study) in vivo micro-CT scans of the distal radius of cynomolgus monkeys were used to monitor changes in architecture over time using a

Table 1.2 Summary of application of bone end-points to toxicology studies

Study type	Markers	Growth+	X-ray	DXA/pQCT		Micro-CT	Histo-morphometry	Bio-mechanics
				In vivo	Ex vivo			
PK/PD	√							
Acute/DRF	√							
28 days	√				√	√		
3 months	√		√	√	√	√	√[a]	√
Chronic	√		√	√	√	√	√	√
Lifetime	√		√		√			
Juvenile	√	√	√	√	√	√	√	√
Neonatal		√	√[b]	√[b]	√[b]			√[b]

DRF dose-range finding, + physical measurements of long bones and crown-rump
[a]Rodent only
[b]Non-rodent only

registration process based on specific anatomical landmarks (Ominsky et al. 2017b). However, these types of applications are normally used in specialized mechanistic studies, not as part of a toxicity study.

Data from biochemical markers, radiography, bone densitometry, histomorphometry, and biomechanical testing are integrated to "tell the story" regarding the mechanism driving any bone changes and whether the outcome was beneficial or adverse to the skeleton. These data are not interpreted in isolation; however, and the body of data from all toxicology study end-points are considered. Not all studies require inclusion of all bone end-points (Table 1.2). All end-points might typically be included in definitive chronic studies, for example, or where changes in vivo were equivocal, and additional end-points such as the biomechanics may be needed to provide a definitive outcome. However, in many instances, data from in vivo end-points (markers and imaging) are sufficient to make a "go/no-go" decision.

The tier approach to the inclusion of bone end-points in preclinical toxicity studies is summarized in Table 1.3. The use of these primary outcome measures in toxicology studies will serve to provide important information regarding skeletal safety. However, regulatory agencies often request additional information to support a submission which may require specialized mechanistic studies or investigation of other aspects of bone quality. Mechanistic studies can be designed to address specific aspects of drug activity on bone and could include techniques such as 5-bromo-2′-deoxyuridine (BrdU) labeling (Mead and Lefebvre 2014) or sophisticated fluorochrome labeling to investigate the chronology of changes (Boyce et al. 2017; Ominsky et al. 2015, Chap. 8). Other tests of bone quality include microscopic, spectroscopic, physical, and chemical techniques to characterize the mineral and collagenous components of bone tissue and are nicely reviewed by Donnelly (2011). These include high-resolution micro-CT scanning (HR-pQCT, quantitative assessment of microarchitecture and measures true tissue mineral density; ionizing radiation), magnetic resonance imaging (MRI, generates 3-D images of bone geometry

Table 1.3 Tier approach to include specialized bone end-points in preclinical toxicology studies

In vivo
Physical measurements, including radiography to assess growth plate closure and abnormalities
Bone densitometry: bone geometry, BMC, BMD
Biochemical markers of bone turnover and/or hormones
Ex vivo
Bone densitometry: bone geometry, BMC, BMD
Histomorphometry: structural and dynamic parameters – mechanistic information
Biomechanical strength testing – ultimate test of bone quality
Other tests to characterize bone quality

and microarchitecture without ionizing radiation), nuclear magnetic resonance imaging (NMR, nonionizing characterization of bone composition including the chemical bonding of bone mineral but no information on the bone matrix), Fourier transform infrared (FTIR) and Raman techniques (characterize bone tissue composition, both mineral and matrix, Boskey and Robey 2013), scanning electron microscopy (SEM, characterizes the morphology and composition of bone surfaces), chemical analyses of collagen cross-links (determines the total amount of collagen and the types of cross-links present), and gravimetric analyses (determines the mineral content of bone tissue based on the ash weight normalized to the dry weight). These techniques have been used to provide valuable information regarding bone quality to further characterize test article effects as part of drug development programs (e.g., see Saito et al. 2015; Ominsky et al. 2017b).

1.4 Considerations for Study Design

1.4.1 Biochemical Markers of Bone Turnover

Biochemical markers are analyzed in blood and urine so can easily be incorporated into any study design for any species, with the exception of neonatal animals (Table 1.2). In a toxicology study, samples for markers can be collected coincident with routine clinical pathology sampling. Optimally, a panel of markers consists of two bone formation markers (osteocalcin (OC), bone-specific alkaline phosphatase (BAP), procollagen type I propeptide (PINP)) and two bone resorption markers (deoxypyridinoline (DPD), C-telopeptides (CTx), N-telopeptides (NTx), tartrate-resistant acid phosphatase 5b (TRACP5b)). Bone formation markers and CTx, NTx, and TRACP5b can be analyzed in serum; DPD, CTx, and NTx can be analyzed in urine. As a minimum, one formation and one resorption marker can be used and may be the only option for test species where validated bone assays may be limited (e.g., pigs). If limited to one of each, then PINP (or osteocalcin if PINP is

not available) (formation) and CTx (resorption, either serum or urine) are recommended (total ALP will also be available as part of the clinical pathology data). Since it is important to understand the net effect of changes in bone formation and bone resorption, use of a single marker is not recommended unless a specific PD marker has been identified. For more information on the markers, the reader is referred to Chap. 5.

For biochemical markers, the most significant limitation is sample volume. Blood sampling in a toxicology study can be dominated by the requirements for assays including clinical pathology, hormones, other PD markers, immunology, pharmacokinetics (PK), and/or sampling for anti-drug antibody (ADA) assessments. Most serum bone marker assays require 75 μL or 150 μL if backup samples are collected. While it may be feasible to analyze multiple markers at a single timepoint in a large animal species study, it is often impractical to do so in rodent studies (notably for mice and juvenile rats) which may require separate subpopulations or use of techniques such as micro-sampling (for relevant assays) to provide samples for all assays. When there is a choice of test system, the rat is recommended for use rather than the mouse to optimize sampling procedures not just for blood and urine, but also bone size for ease of in vivo imaging and postmortem end-points. However, use of the mouse as a test system is unavoidable where the drug target is not appropriate for the rat, driving the need to adapt procedures for blood and urine collection and analysis. The development of Luminex-based assays for several bone markers has significantly reduced the sample volume requirements making the mouse a more feasible test system, as well as increasing the scope of assays that can be performed for the rat. Another strategy adopted when sample volume is limited is to collect the minimal volume to perform a single-point determination for each marker and retain a single backup sample for repeat analyses of any assays that fail.

For bone resorption markers, no special collection procedures are required for urine for the rat or large animals. Following an overnight fast, collection in jars from the cage pan, without ice, is adequate if fecal contamination is avoided. Direct collection from the bladder by catheterization or cystocentesis is an option for the monkey and dog but collection requires animals to be sedated which may not be appropriate in a toxicology study setting. The advantage of collecting a urine sample by catheterization is to reduce variability due to potential bacterial growth. For monkeys, the anesthetic used consists of an intramuscular injection of a cocktail of glycopyrrolate, ketamine, and dexmedetomidine. Atipamezole is used to reverse the anesthesia once the urine sample is obtained. The catheterization procedure is performed in the animal room following aseptic-like procedures. For males, the insertion of the catheter is done very slowly using a catheter of adequate length to prevent the creation of loops which would not allow the removal of the catheter from the urethra. Alternatively, following an overnight fast, a relatively clean urine sample can be obtained from the cage pan early morning during a 4-h collection period with water deprivation. Urine collection for the mouse historically has been avoided although collection in metabolic cages (as used for the rat) has shown some success. A mouse study needs to be adequately powered to compensate for (potentially) several missing samples, to provide adequate data for interpretation. Although yet

to be validated for use with bone markers, a relatively new technique using LabSand® allows urine collection from group-housed mice over a 5-h period. LabSand® is a hydrophobic, commercially available sand that keeps the urine afloat. The LabSand® replaces the cage bedding, animals are free to move around the cage, and urine drops are collected using a pipette (Doyle et al. 2017).

The conditions for sample collection are important and need to be consistent. Circadian rhythms, hormonal status, stress, and diet can influence bone markers. In a regulated preclinical study, it is relatively easy to control diet and environmental conditions (light cycle, temperature, and humidity) and dose administration. Samples should be collected at the same time of day and under the same conditions (normally fasted) throughout the study. To ensure samples are obtained consistently, an early morning collection is recommended, between 8 and 10 a.m., following an overnight fast. Alternatively, samples can be collected in the afternoon, following several hours of food deprivation, rotating the groups throughout the collection period. Fruits normally provided to monkeys as part of an enrichment program should not be given for approximately 2 days prior to sampling for bone markers. As a non-certified food supplement, the type and amount of fruit provided can vary from one sampling occasion to another and even among animals in the same study population; therefore, this variable is removed in an attempt to control sampling conditions as much as possible.

Establishing a PK/PD profile is not recommended for all test articles but it can provide information regarding the optimal time for sampling for bone markers and may identify a specific PD marker that can be used in subsequent studies (e.g., see Ominsky et al. 2010). Data derived using a strict sampling regime can be used with confidence and compared cross-sectionally with controls or across time. Toxicology studies typically use young animals with skeletal growth ongoing; as the skeleton matures, bone marker levels decline. Age-related decreases in bone markers are evident in most studies and do not interfere with data interpretation. Historical control data are of great value when compiled for each species, sex, and age-range, particularly for non-rodent species. Samples for bone markers are rarely obtained in pediatric studies from very young (neonatal) animals (unless a decrease is expected) because marker levels are high and more variable than “older” animals. Sample volume is often limiting and removal of offspring from their mother is usually not recommended for data that may have limited value. In longer-term rodent juvenile toxicology studies, samples can be obtained from around 21 days of age, or when animals reach ages similar to those at the start of routine toxicology studies, and can provide important data.

Sample collection for bone marker analysis is normally coincident with sampling for routine clinical pathology so that data from clinical pathology parameters such as calcium, phosphorus, and alkaline phosphatase are available for interpretation. Sample collection should be scheduled around the time scan data is acquired so that marker levels can be correlated with any changes in bone mass. Samples for clinical pathology and bone markers should ideally be collected before fluorochrome labels are injected (see Sect. 4.4) or at least 2 days afterward to allow sufficient clearance of the labels. The presence of fluorochrome labels may interfere

with some assays. Samples for markers typically are not required pre-study at baseline for rats because studies are normally well powered (n = 8 to 10/sex/group) and rat populations are relatively homogeneous with respect to marker levels. In rat studies with a large population, sampling for marker analysis may be restricted to a portion of the population (usually 10/sex/group). Because marker levels are relatively homogeneous, animals lost during the study can be readily replaced from others within the same group. Similarly, where sample volume is limited as in pediatric and mouse studies, the use of dedicated subpopulations for various activities is an excellent strategy. Data derived at different timepoints from separate subpopulations are then collated for interpretation. Micro-sampling may be an optional technique for some assays. For monkeys or dogs, marker levels can be variable within a population and group sizes are small. The study design therefore should include adequate sampling pre-study (two occasions recommended) to establish baseline levels for individual animals that can be monitored across time. Bone markers do fluctuate despite rigorous control of sampling conditions, so multiple occasions are useful. Deriving the percent change from average baseline levels may be used for interpretation in some instances, but percent values can often be misleading, especially where the working range of values for the assay is numerically low.

1.4.2 Environmental Conditions and Diet

Environmental conditions are recognized as becoming increasingly important as factors that influence skeletal activity. Diurnal variation is controlled using a consistent light cycle. Room temperature has recently come into focus as an important factor affecting bone mass. Female mice housed under thermoneutral conditions do not show the age-related cancellous bone loss that occurs in mice housed at 22 °C (Iwaniec et al. 2016). The beneficial effects of thermoneutral housing on cancellous bone were associated with decreased Ucp1 gene expression in brown adipose tissue, increased bone marrow adiposity, higher rates of bone formation, higher expression levels of osteogenic genes, and locally decreased bone resorption. Diet and the biotome also affect bone mass (Steves et al. 2016). Manipulating the diet, such as use of low/high fat diets or low/high calcium diets, influences bone metabolism. Primate chow also contains phytoestrogens which may influence the skeleton in conditions of estrogen deprivation (as occurs when testing aromatase inhibitors, for example, or in ovariectomized animals). Tight control of diet and environmental conditions for studies with bone assessments is therefore important.

Stress and the effects of ACTH and glucocorticoids on bone are well documented (Kaltsas and Makras 2010). Social housing vs single housing conditions influence stress levels; therefore, differences in housing may influence the study outcome. Stress associated with laboratory conditions is usually well controlled where experienced staff perform the dosing and animal husbandry procedures. The drug-induced toxicity that can result in some studies however cannot be controlled, and special attention should be paid to the pathologist's observations of lesions that may

be attributed to stress. Stress can influence hormone levels in general which can have profound effects on bone. For additional perspective, the reader is referred to Chap. 10 which addresses the influence of pituitary hormones and Chap. 14 for the influence of the intestinal microbiota.

1.4.3 Radiography and Bone Densitometry

The reader is referred to Chap. 6 for more in-depth information regarding skeletal imaging and to Chap. 3 for specific information on the use of young animals to test therapeutics intended for pediatric populations. The following section highlights some of the considerations required for inclusion of these techniques in preclinical toxicology studies.

Radiography and bone densitometry require animals to be anesthetized if performed in-life; the risk associated with this needs to be carefully considered for each study. Isoflurane is the anesthetic of choice for most species and is safe to use. Needless to say, animals that are sick or have overt clinical signs that may be compromising should not undergo anesthesia. Evaluation of excised bones may be an option. As mentioned elsewhere, the bone mass (BMD) of rat bones is normally relatively homogeneous within a population eliminating the need for pre-study baseline scanning. Within the context of a short-term study, up to 28 days duration, scanning of excised rat bones is normally adequate (Table 1.2). Studies 28 days or longer benefit from acquisition of in vivo scan data at intervals during the study. Suggested intervals would be around half way through a 3-month rat study and at 3-month intervals for studies 6 months or longer. Similar intervals are suggested for monkeys and dogs but with large animals it is necessary to obtain pre-study baseline scan data. Data obtained during the treatment period are calculated as the percent change from baseline (pretreatment) for each individual animal to adjust for the variability in bone densitometry parameters seen in large animal species. Non-rodent toxicology studies also have small group sizes which limits the power to obtain robust cross-sectional data.

Radiography is an invaluable tool in studies requiring an assessment of bone growth and is normally combined with more frequent physical measurements of limbs or crown rump in neonatal or juvenile toxicology studies (Table 1.2). The need for anesthesia limits the use of radiography in very young animals. In rat neonatal or juvenile toxicology studies, acquisition of X-rays and bone densitometry scans is typically initiated at around 21 days of age, although animals as young as 14 days of age have been used. If dosing was initiated at an earlier age, then this becomes the first timepoint that meaningful data can be acquired. For dogs (Robinson et al. 2012), in vivo radiographs and limited bone densitometry have been performed as early as 1 day of age. Data acquisition is limited at this very young age by inadequate bone consolidation, restricting bone densitometry to DXA scanning of the whole body. In dogs, regional DXA scans of the lumbar spine and femur become feasible by 2–3 months of age. At this age, scanning using pQCT

also becomes feasible since animals can endure a longer anesthesia time. Following in utero exposure of a bone drug (denosumab), ex vivo radiography and bone densitometry were used in part to characterize the skeletal phenotype in infant cynomolgus monkeys at birth (Boyce et al. 2014).

Site selection for data acquisition is driven by the need to provide clinically translatable data and the practicality associated with obtaining these data. The sites considered most at risk for fracture in humans are the spine, hip, and forearm (radius). Fracture incidence data is acquired in clinical trials for vertebral and non-vertebral fractures, with a separate analysis for the incidence of hip fracture, a site considered associated with the greatest morbidity. Agency guidelines for the testing of compounds to treat or prevent osteoporosis therefore recommend evaluation of these specific sites for efficacy in appropriate osteopenic animal models, typically the rat and monkey (Japanese MHLW 1999; EMA 2007; FDA 2016). These preclinical bone quality studies are designed to mimic the end-points used in clinical trials as much as possible. Bone densitometry assessments were therefore developed to focus on assessments of the spine, hip, and radius. Use of these established sites has subsequently been adapted for general use in safety studies and applied to other species including the dog and rabbit. In humans, a clinical diagnosis of osteoporosis and osteopenia is made using DXA BMD, and DXA remains the only bone densitometry technique approved by the FDA. In preclinical research DXA is therefore an important clinically translatable tool and is associated with several advantages and limitations (Blake and Fogelman 2008; Bolotin 2007). DXA can provide information on large areas of the skeleton such as the spine, radius, and femur, as well as the whole body, and can provide measures of whole body lean and fat mass. However, DXA provides a two-dimensional areal measurement of bone where bone size and positioning can affect outcome (BMD) measures, and it cannot discriminate between trabecular and cortical bone. In vivo DXA BMD measurements are dependent upon surrounding soft tissue; therefore, changes in body composition can impact BMD values whether bone is directly affected or not. Use of pQCT provides a three-dimensional bone density assessment, and complementary data which can compensate for some of the limitations of DXA, as well as facilitate DXA data interpretation. Peripheral QCT can provide separate analyses of the trabecular and cortical bone compartments and important measures of bone geometry. The main limitation of pQCT is that data is acquired from single or multiple images (scan slices) through a cross-section of bone at a peripheral site, normally the proximal tibia or distal radius, metaphysis, and diaphysis. To acquire in vivo pQCT scans, the limb is placed within a gantry or opening. The limbs of some animals, such as the adult dog thigh and obese rat hind limb, cannot be positioned appropriately for scanning; thus for the dog, data acquisition is normally restricted to the radius. Larger regions can be scanned in vivo, including vertebrae and the hip, but in most laboratory settings the larger equipment required to do this is not available. Body composition measures can also be obtained with pQCT.

To address the frequently asked question of which bone densitometry technique to use in a preclinical safety study, both modalities (DXA and pQCT) are recommended. However, pQCT is often recommended alone because of the ability to