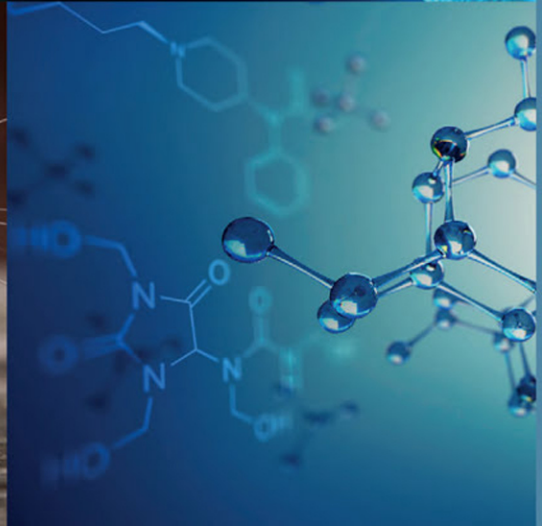
EDITED BY

RAMESH C. GUPTA

SECOND EDITION

# BIOMARKERS IN TOXICOLOGY



# BIOMARKERS IN TOXICOLOGY

## SECOND EDITION

*Edited by*

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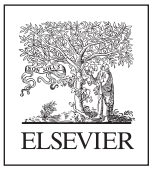


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*This book is dedicated to my daughter Rekha, wife Denise,*
*and parents the late Chandra and Triveni Gupta.*

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# Foreword

The first edition of *Biomarkers in Toxicology*, edited by Ramesh Gupta, was published in 2014. The whole area of biomarkers, not only in toxicology, is rapidly developing, partly because of the availability of highly sophisticated analytical equipment, and so the second edition of this book is greatly welcomed. The second edition contains 12 new chapters, and most of the rest have been updated.

Merriam Webster defines a biomarker as a distinctive biological or biologically derived indicator (as a metabolite) of a process, event, or condition (as, for example, aging, disease, or oil formation). There are other definitions, for example, in Environmental Health Criteria 222 Biomarkers. In Risk Assessment http://www.inchem.org/documents/ehc/ehc/ehc222.htm#1.0 biomarkers are defined thus "A biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease." The subject of the present book is biomarkers in toxicology, but it should be remembered that biomarkers include substances used in the detection of numerous diseases, including the autoimmune diseases, which are not generally thought to be toxicological in origin. However, biomarkers are crucial to toxicology and allied disciplines such as epidemiology and risk assessment.

The earliest toxicological biomarkers of exposure date from before precise analytical techniques were available and include the Kayser−Fleischer ring (described 1902/3), usually indicative of copper accumulation in the cornea in cases of Wilson's disease, as well as lead lines in the gums associated with lead toxicity. The cherry red color noted as a (rather unreliable) clinical sign in carbon monoxide poisoning may also be described as a biomarker.

One of the earliest biomarkers relying on biochemical analytical techniques was measurement of cholinesterase activity, initially whole blood cholinesterase and later plasma pseudocholinesterase (butyrylcholinesterase) and red blood cell acetylcholinesterase. Cholinesterase measurements were introduced at defense laboratories after World War II as a screening test for excessive exposure to organophosphate compounds: a 20% depression in activity was considered to mandate cessation of exposure of individuals to organophosphate nerve agents (the basis of the 20% figure is obscure, but seemed protective). Pseudocholinesterase and red blood cell acetylcholinesterase measurements now have numerous uses in worker protection, clinical diagnosis of poisoning, and human and experimental animal studies (including regulatory ones) in relation to the use of organophosphate and other anticholinesterase pesticides. Since World War II biomarkers of toxicity have ballooned in importance and number in worker and consumer protection and clinical and experimental toxicology and are also widely used in regulation of chemicals in animal and, less commonly, human experimental studies. Toxicological biomarkers are also used in allied disciplines, for example, epidemiology, and may be used to estimate loads of exposure in populations being investigated.

In toxicology, biomarkers are often divided into biomarkers of exposure, of effect, and of susceptibility, and all of these are dealt with in this book, which is extremely wide-ranging. The book has an initial introductory part, including discussion of rodent, nonhuman primate, and zebrafish and *Caenorhabditis elegans* models for toxicological testing. There are two new chapters in this part: firstly, *Drosophila melanogaster, Eisenia fetida,* and *Daphnia magna* for toxicity testing and biomarkers and secondly, adverse outcome pathways and biomarkers.

Part II, systems toxicity biomarkers, comprises chapters on biomarkers of toxicity in relation to all important organs and organ systems. There is an additional chapter on reproductive and developmental toxicity biomarkers, and another on ototoxicity biomarkers. Part III, renamed chemical agents, solvents, and gases toxicity biomarkers, deals with biomarkers in relation to the toxicity of specific groups of compounds and comprises chapters on pesticides, as well as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), brominated flame retardants, polycyclic aromatic hydrocarbons (PAHs), bisphenol A, melamine and cyanuric acid, and metals; there is a very useful chapter on biomarkers of chemical mixture toxicity. Part IV (biotoxins biomarkers) has three chapters on, respectively, freshwater cyanotoxins, mycotoxins, and poisonous plants: biomarkers for diagnosis (of poisoning). Part V covers pharmaceuticals and nutraceuticals, with chapters on drug toxicity biomarkers and nutriphenomic biomarkers together with a new chapter on biomarkers of toxicity for dietary ingredients contained in dietary supplements. Part VI covers nanomaterials and radiation, with two chapters, one on biomarkers of exposure and effect of engineered nanomaterials and the other on biomarkers of exposure and effects of radiation.

Part VII is entitled carcinogens biomonitoring and cancer biomarkers and contains six chapters. These are on biomonitoring exposures to carcinogens, genotoxicity biomarkers, epigenetic biomarkers in toxicology, breast cancer biomarkers, pancreatic and ovarian cancer biomarkers, and prostate cancer biomarkers. Part VIII is called disease biomarkers and deals with biomarkers of Alzheimer's disease, biomarkers of Parkinson's disease, biomarkers for drugs of abuse and neuropsychiatric disorders: models and mechanisms, osteoarthritis biomarkers, pathological biomarkers in toxicology and oral pathology biomarkers. Of these, the chapters on osteoarthritis and oral pathology are new. Part IX is called special topics. This part of the book contains chapters on biomarkers of mitochondrial dysfunction and toxicity, biomarkers of blood-brain barrier dysfunction, biomarkers of oxidative/nitrosative stress and neurotoxicity, cytoskeletal disruption as a biomarker of developmental neurotoxicity, membrane transporters and transporter substrates as biomarkers for drug pharmacokinetics, pharmacodynamics, and toxicity/adverse events, and citrulline: pharmacological perspectives and role as a biomarker in diseases and toxicity. Of these chapters, that on the blood-brain barrier dysfunction is new and is particularly welcome as the blood-brain barrier is very important in protecting the central nervous system against toxicants. The last part of the book is on applications of biomarkers. It contains three new chapters: biomarkers detection for toxicity testing using microarray technology, metabolomics, and proteomics. Also there are chapters on transcriptomic biomarkers, percellome toxicogenomics, biomarkers in computational toxicology, biomarkers in biomonitoring of xenobiotics and biomarkers in toxicology, risk assessment, and environmental chemical regulations.

The 67-chapter book has an outstanding array of authors from the United States, Canada, Denmark, Finland, Greece, India, Italy, Japan, Portugal, Romania, and Spain. Professor Gupta deserves our gratitude for assembling such a distinguished group of experts to produce so comprehensive a book on this rapidly growing and very important field.

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CHAPTER

# 1

# Introduction

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Biomarkers can broadly be defined as indicators or signaling events in biological systems or samples of measurable changes at the molecular, biochemical, cellular, physiological, pathological, or behavioral levels in response to xenobiotics. The Biomarkers Definitions Working Group of the National Institutes of Health (NIH) has defined the biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes or pharmacological responses to a therapeutic agent." In the field of toxicology, biomarkers have been classified as markers of exposure, effect, and susceptibility. Measurement of biomarkers reflects the time course of an injury and provides information on the molecular mechanisms of toxicity. These biomarkers provide us the confidence of accurate diagnosis, prognosis, and treatment. The biomarkers of early chemical exposure can occur in concert with biomarkers of early disease detection, and that information aids in avoiding further chemical exposure and in strategic development of a novel treatment, including personalized medicine (i.e., treating the patient, and not the disease). In essence, with the utilization of specific biomarkers, an ounce of prevention can be worth a pound of treatment.

Biomarkers are used in drug development, during preclinical and clinical trials, for efficacy and safety assessment. Biomarkers can reveal valuable information regarding diagnosis, prognosis, and predict treatment efficacy or toxicity; serve as markers of disease progression; and serve as auxiliary endpoints for clinical trials (Stern et al., 2018), with the ultimate goal of delivering safe and effective medicines to patients (Lavezzari and Womack, 2016: Gerlach et al., 2018). In addition, a biomarker in drug development should be ethically acceptable (Hey, 2017). Safety biomarkers can be used to predict, detect, and monitor drug-induced toxicity during both preclinical studies and human clinical trials. Developing and validating highly sensitive methods for measurement of biomarkers and understanding the resultant data are complex processes that require a great deal of time, effort, and intellectual input. Furthermore, understanding drug metabolism seems essential in some cases, as the metabolite of a drug can be used as a biomarker, and the drug and/or its metabolite has to be patented by the United States Patent Office and by a similar governmental office/agency in other countries. In the past, many drugs were developed with biomarker assays that guided their use, and this trend is likely to continue in the future for drug discovery and development. With the judicious use of biomarkers, as in evidence-based medicine, patients are most likely to benefit from select treatments and least likely to suffer from their adverse effects. On the contrary, utilization of a bad biomarker can be as harmful to a patient as a bad drug. Therefore, biomarkers need to be validated and evaluated by an accredited laboratory, which participates in a proficiency testing program, to provide a high level of confidence to both clinicians and patients.

In the toxicology field, biomarkers should be specific, accurate, sensitive, validated, biologically or clinically relevant, and easy and fast to perform to be useful as predictive tools for toxicity testing and surveillance and for improving quantitative estimates of exposure and dose. Therefore, biomarkers are utilized for biomonitoring data that are useful in a variety of applications, from exposure assessment to risk assessment, management, and regulations (Ganzleben et al., 2017).

In the early 1990s, Dr. Maria Cristina Fossi from the University of Siena, Italy, emphasized the approach for the development and validation of nondestructive biomarkers over destructive biomarkers in the field of toxicology. She described the ideal biomarker as being measurable in readily available tissues or biological products and obtainable in a noninvasive way; related

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to exposure and/or degree of harm to the organism; directly related to the mechanism of action of the contaminants; highly sensitive with techniques that require minimal quantities of sample and are easy to perform and cost-effective; and suitable for different species.

The development and validation of new techniques in the laboratory may provide the basis for a valuable field method. But, before a new biomarker's application, some basic information is required, such as dose–response relationships, and biological and environmental factors, which can influence the baseline values of responses. It is important to mention that, when dealing with a biochemical or metabolic biomarker, species differences can be the biggest challenge for any toxicologist.

Biomarkers have applications in all areas of toxicology, especially in the fields of pesticides, metals, mycotoxins, and drugs. In the case of veterinary toxicology, biomarkers of plant toxins deserve equal attention. Farmers, pesticide application workers, and greenhouse workers are exposed to pesticides by direct contact and their family members can be exposed via secondhand exposure. Measurement of residues of pesticides, and their metabolites and metals in urine, serves as the most accurate and reliable biomarkers of exposure in agriculture, industrial, and occupational safety and health settings. Recent evidence suggests that in utero or early life exposure to certain pesticides, metals, and other environmental contaminants may cause neurodegenerative (Alzheimer's, Parkinson's, schizophrenia, Huntington's, ALS, and others) and cardiovascular diseases, diabetes, and cancer later in life. In these diseases and many others, specific and sensitive biomarkers play important roles in early diagnosis, and this can serve as the cornerstone for timely therapeutic intervention.

Mycotoxin-related toxicity, carcinogenesis, and other health ailments are encountered in man and animals around the world. In developing countries, where regulatory guidelines are not strictly followed, adverse health effects (especially reproductive and developmental effects) are devastating. In these scenarios, early biomarkers of exposure play a pivotal role in avoiding further exposure to the contaminated food/feed and thus safeguard human and animal health.

With the current knowledge of system biology, proteomics, metabonomics, toxicogenomics, and various mathematical and computational/chemometric modelings, undetectable biomarkers can be discovered and these biomarkers can predict how tissues respond to toxicants and drugs and/or their metabolites, and how the tissue damage and repair processes compromise the tissue's function. Imaging and chemometric biomarkers are of greater sensitivity and carry more information than conventional biomarkers, as they detect (1) low levels of chemical exposure (exposure biomarker) and (2) an early tissue response (endogenous response biomarker). The priority will always be for the development of a noninvasive approach over an invasive approach, and nondestructive biomarkers over destructive biomarkers, but this may not be possible in all cases.

In 2011, the Joint SOT/EUROTOX Debate proposed that "biomarkers from blood and urine will replace traditional histopathological evaluation to determine adverse responses," identifying and comparing the strengths and limitations of histopathology with serum and urine biomarkers. Unlike histopathological techniques, blood and urine biomarkers are noninvasive, quantifiable, and of translational value. Of course, the complete replacement of histopathological biomarkers with blood and urine may not be possible in the near future, as in some instances histopathological biomarkers will still be used because of recent developments in invaluable molecular pathology techniques.

For the quest of developing the most sensitive and reliable biomarkers, integration of novel and existing biomarkers with a multidisciplinary approach appears fruitful. Furthermore, a multibiomarker approach seems more informative and accurate than a single biomarker approach. By now, microRNAs (miRNAs) have been well recognized as reliable and robust biomarkers for early detection of diseases, birth defects, pathological changes, cancer, and toxicities (Quiat and Olson, 2013; Wang et al., 2013; Bailey and Glaab, 2018). Because they are stable in biofluids, such as blood, there is rapidly growing interest in using miRNAs as diagnostic, prognostic, and predictive biomarkers, and the outlook for the clinical application of miRNA discoveries is promising, especially in molecular medicine. Soon, incorporating pharmacological and toxicological targeting of miRNAs into the development of innovative therapeutic strategies will be routine. Still, more innovative biomarkers need to be developed that will be highly sensitive (biotechnology-based techniques), require minimum quantities of sample, and will promise high-throughput screening.

At the recent meetings of the Society of Toxicology, the EUROTOX, and International Congress of Toxicology, a large number of toxicologists emphasized the importance of biomarkers in health, disease, and toxicity. Accordingly, *Biomarkers in Toxicology*, second edition has been prepared to meet the challenges of today's toxicologists, pharmacologists, environmentalists, and physicians in academia, industry, and government. This reference book is of particular interest to those in governmental agencies, such as NIH, USEPA, USFDA, USDA, NIOSH, OSHA, CDC, REACH, EFSA, etc. This is the most comprehensive biomarkers book to date as it covers every possible aspect of exposure, effects, and susceptibility to chemicals. There are many novel topics

in this volume that are not covered in any previous book. This edition identifies and establishes the most sensitive, accurate, unique, and validated biomarkers that can be used as indicators of exposure and effect(s) of chemicals, and chemical-related long-term diseases, such as cardiovascular, metabolic and neurodegenerative diseases, and cancer. Sixty-seven chapters are organized under eight sections with a user-friendly format, and each chapter is enriched with current literature and references for further reading. This book begins with general concepts of toxicity and safety testing and biomarker development using various animal and animal alternative models, adverse outcome pathways, followed by biomarkers of system/organ toxicity, chemicals, solvents, gases, and biotoxins. There are several chapters on biomarkers of pharmaceuticals, nutraceuticals, petroleum products, chemical mixtures, radiation, engineered nanomaterials, epigenetics, genotoxicity, and carcinogens. In the disease section, chapters cover the biomarkers of Alzheimer's, Parkinson's, neuropsychiatric disorders, osteoarthritis, and some other pathological conditions. Under special topics, chapters are included on mitochondrial dysfunction and toxicity, the blood−brain barrier, oxidative/nitrosative stress, developmental neurotoxicity, miRNAs as indicators of tissue injury, and citrulline in diseases and toxicity. Lastly, a large number of chapters are dedicated to the application of biomarkers in toxicology, including the latest strategies and technologies in the development of biomarkers, biomarkers in drug development, safety evaluation, and toxicity testing and integration of biomarkers in biomonitoring of chemical exposure and risk assessment, especially in the context of industrial, environmental, and occupational medicine and toxicology.

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CHAPTER

# 2

# Rodent Models for Toxicity Testing and Biomarkers

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## INTRODUCTION

Three rodent species are widely used in toxicology: the rat, the mouse, and the hamster. Two of these, the rat and mouse, are the most widely used in experimental biology and medicine. These have formed the basis for exploring the efficacy of drugs and for the identification and evaluation of toxicities associated with exposure to drugs, industrial and agricultural chemicals, and understanding the mechanisms of their toxicity since toxicology became an identified discipline.

A large (and growing) set of biomarkers are known for use in identifying and determining the relative (and relevant) risks to humans or other target species. These include:

- Body weights
- Clinical pathology (hematology)
- Clinical chemistry
- Organ weights
- Gross histologic changes at necropsy
- Immunogenicity
- Microscopic evaluation of tissues
- Changes in physiologic functions and electrophysiology
- Effects on specific genomic markers

Biomarkers are measurements of test model (animal) parameters that can provide important quantitative data about the biological state of the test model, which are predictive of effects in humans. These biomarkers in toxicology are preferably shared by both test animals and humans and in a manner that the relationship of findings in one species to another is known.

Accordingly, we will proceed to understand the current uses of these three rodent species as predictive models for effects in humans, as well as how they are measured and what their normal ranges are.

As these potential pieces of data are overviewed and considered, it is important to remember that each of these biomarkers is a part of the overall picture as to what the model is predicting as per potential adverse effects in humans. Meaningful safety assessment requires that all the data be incorporated in an integrated safety assessment. Because dose/toxicodynamic relationships will vary with level of exposure of test animals, it is also necessary that multiple (traditionally at least three) "dose" levels be evaluated.

The picture becomes both more complex but also clearer as to relevance as new biomarkers are identified and became understandable. These include proteomics (Amacher, 2010), new clinical chemistry parameters, immune system responses, and real time functional physiologic system measurements by telemetrized instrumentation (Gad, 2013).

Table 2.1 (Gad, 2013) presents the current most relevant associations of biomarkers with renal and liver toxicity, whereas Table 2.2 (adopted from Gad, 2013) presents an overview of the association between classical clinical chemistry parameters and specific target organ toxicities (Table 2.3). Table 2.4 summarizes causes associated with hematological findings in the rat.

Over the last 10 years, diligent efforts under the rubric of the Critical Path Initiative have led to the identification of a more specific set of clinical chemistry biomarkers for key potential target organs.

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| | | |
|---|---|---|
| Heart | Troponins | Zethelius et al. (2008) |
| Kidney | KIM-1, Albumin, Beta-2-Microglobulin | Hoffmann et al. (2010), Ozer et al. (2010), and Vaidya et al. (2010) |
| Liver | DILI (Drug Induced Liver Injury)l ALT, BUN, coagulation factor | Shi et al. (2010) |

Other recently identified biomarkers for specific targets include:

1. *Mitochondrial Dysfunction*. Increased uptake of calcium (because ATP depletion) by mitochondria activates phospholipases, resulting in accumulation of free fatty acids. These cause changes in the permeability of mitochondrial membranes, such as the *mitochondrial permeability transition*.
2. *Progressive Loss of Phospholipids*. Increased degradation by endogenous phospholipases and inability of the cell to keep up with synthesis of new phospholipids (reacylation, an ATP-dependent process).
3. *Cytoskeletal Abnormalities*. Activated proteases lyse cytoskeletal elements and cell swelling causes detachment of cell membrane from cytoskeleton; stretching of the cell membrane results in increased membrane damage.
4. *Reactive Oxygen Species*. Produced within the cell by infiltrating neutrophils and macrophages, especially after restoration of blood flow to an area (reperfusion injury). Cell injury triggers release of a number of inflammatory cytokines and chemokines that amplify the host immune response and attract neutrophils to the site.
5. *Lipid Breakdown Products*. Unesterified free fatty acids, acyl carnitine, and lysophospholipids. These have a detergent effect on membranes and may exchange with membrane phospholipids, causing permeability changes.

**TABLE 2.1** Classic Associations in Toxicology

| Liver Toxicity | Renal Toxicity |
|---|---|
| Increased plasma activity of liver marker enzymes, e.g., alanine and aspartate aminotransferases | Increased water consumption and urine volume. Urine parameters may change, e.g., enzymes and cellular debris. |
| Decreased plasma total protein concentration | Increased plasma concentrations of urea and creatinine. Proteinuria. |
| Increased coagulation times due to decreased synthesis of coagulation factors | Severe renal toxicity may lead to decreased erythrocyte parameters due to effects on erythrocyte synthesis |
| Increased liver weight due to enzyme induction or accumulation of lipid or glycogen | Increased kidney weight |
| Change in color or size at necropsy | Change in color or size at necropsy |
| Histological findings such as necrosis or centrilobular hypertrophy due to enzyme induction | Histological change, e.g., basophilic tubules or necrosis, papillary necrosis, or glomerular changes. |

# THE RAT

## Use in Toxicological Research

Ideally, safety testing of products intended for use in humans, or to which humans could be exposed, should be done in humans. The data from humans would apply without reservation to complex human physiology and cellular/biochemical mechanisms and human risk assessment. Unfortunately, humans cannot be used for this purpose. Therefore, the choice of an appropriate species for toxicology studies should be based on a comparison of the pharmacokinetics, target pharmacodynamics, and metabolism of the test compound in different laboratory species and man. In the absence of this data, this choice is often based on practicality and economics. The rat has become a species of choice because of the metabolic similarities, as well as their small size, relatively docile nature, short life span, and short gestation period. The extensive use of the rat in research has led to the development of a large historical database of their nutrition, diseases, and general biology.

### *Characteristics*

Although the rat is a species of choice in toxicology research because of the many physiological similarities and anatomical characteristics, differences exist that must be considered when designing and conducting studies with this animal. Rats are obligate nose breathers; as such an inhaled test material is subject to nasal filtration and absorption. The placenta is considerably more porous in the rat. This difference may increase the chance of fetal exposure to an administered test material or increase the overall level of fetal exposure to an administered test material. The overall distribution of intestinal microflora is different in the rat, which may lead to differences in the metabolism of an orally administered test material. These and other differences in the rat may lead to positive signs of toxicity to a test material that may not be present in a different species. There are

**TABLE 2.2** Association of Changes in Biochemical Parameters With Actions at Particular Target Organs (Gad, 2013)

| Parameter | Blood | Heart | Lung | Kidney | Liver | Bone | Intestine | Pancreas | Notes |
|---|---|---|---|---|---|---|---|---|---|
| Albumin | | | | ↓ | ↓ | | | | Produced by the liver; very significant reductions indicate extensive liver damage |
| ALP | | | | | ↑ | ↑ | ↑ | | Elevations usually associated with cholestasis; bone alkaline phosphatase tends to be higher in young animals |
| ALT (formerly SGPT) | | | | | ↑ | | | | Elevations usually associated with hepatic damage or disease |
| AST (formerly SGOT) | | ↑ | | ↑ | ↑ | | | ↑ | Present in skeletal muscle and heart and most commonly associated with damage to these |
| Beta-2-Microglobulin | | | | ↑ | | | | | |
| Bilirubin (total) | ↑ | | | | ↑ | | | | Usually elevated due to cholestasis, due to either obstruction or hepatopathy |
| BUN | | | | ↑ | ↓ | | | | Estimates blood filtering capacity of the kidneys; does not become significantly elevated until the kidney function is reduced 60%–75% |
| Calcium | | | | ↑ | | | | | Can be life threatening and result in acute death |
| Cholinesterase | | | | ↑ | ↓ | | | | Found in plasma, brain, and RBC |
| CPK | | ↑ | | | | | | | Most often elevated due to skeletal muscle damage but can also be produced by cardiac muscle damage; can be more sensitive than histopathology |
| Creatinine | | | | ↑ | | | | | Also estimates blood filtering capacity of kidney as BUN does |
| Glucose | | | | | | | | ↑ | Alterations other than those associated with stress uncommon and reflect an effect on the pancreatic islets or anorexia |
| GGT | | | | | ↑ | | | | Elevated in cholestasis; this is a microsomal enzyme, and levels often increase in response to microsomal enzyme induction |
| HBDH | | ↑ | | | ↑ | | | | – |
| KIM-1 | | | | ↑ | | | | | |
| LDH | | ↑ | ↑ | ↑ | ↑ | | | | Increase usually due to skeletal muscle, cardiac muscle, or liver damage; not very specific |
| Protein (total) | | | | ↓ | ↓ | | | | Absolute alterations usually associated with decreased production (liver) or increased loss (kidney); can see increase in case of muscle wasting (catabolism) |
| SDH | | | | | ↑↓ | | | | Liver enzyme that can be quite sensitive but is fairly unstable; samples should be processed as soon as possible |
| Trophonin | | ↑ | | | | | | | |

*ALP*, alkaline phosphatase; *BUN*, blood urea nitrogen; *CPK*, creatinine phosphokinase; *GGT*, gamma glutamyl transferase; *HBDH*, hydroxybutyric dehydrogenase; *LDH*, lactic dehydrogenase; *RBCs*, red blood cells; *SDH*, sorbitol dehydrogenase; *SGOT*, serum glutamic oxaloacetic transaminase (also called AST [aspartate amino transferase]); *SGPT*, serum glutamicpyruvic transaminase (also called ALT [alanine amino transferase]); ↑, increase in chemistry values; ↓, decrease in chemistry values.

**TABLE 2.3** Liver Enzymes

| "Liver Enzyme" | Nomenclature | Plasma-Tissue Sources | Cellular Location |
|---|---|---|---|
| AST (SGOT) | Aspartate Aminotransferase | Liver, Heart, Skeletal Muscle, Kidney, Brain, RBCs | Mitochondria, Cytoplasm |
| ALT (SGPT) | Alanine Aminotransferase | Mostly liver, Heart, Skeletal Muscle | Cytoplasm |
| Alk Phos (AP) | Alkaline Phosphatase | Bile ducts, GI tract, Bone, Placenta | Membranes |
| GGT | Gamma-glutamyl transferase | Liver, Kidney, Heart | Membranes |
| GDH | Glutamate Dehydrogenase | Liver, Kidney Skeletal Muscle | Mitochondria |
| SDH | Sorbitol Dehydrogenase | Mostly liver | Cytoplasm |
| LDH | Lactate Dehydrogenase | Heart, Skeletal Muscle, RBCs, Lung, Liver, All tissues | Cytoplasm |

*RBC*, red blood cell.

**TABLE 2.4** Some Probable Conditions Affecting Hematological Changes (Gad, 2013)

| Parameter | Elevation | Depression | Parameter | Elevation | Depression |
|---|---|---|---|---|---|
| Red blood cells (RBCs) | **1.** Vascular shock<br>**2.** Excessive diuresis<br>**3.** Chronic hypoxia<br>**4.** Hyperadreno corticism | **1.** Anemias<br>**a.** Blood Loss<br>**b.** Hemolysis<br>**c.** Low RBC production | Platelets | | **1.** Bone marrow depression<br>**2.** Immune disorder |
| Hematocrit | **1.** Increased RBC<br>**2.** Stress<br>**3.** Shock<br>**a.** Trauma<br>**b.** Surgery<br>**4.** Polycythemia | **1.** Anemias<br>**2.** Pregnancy<br>**3.** Excessive hydration | Neutrophils | **1.** Acute bacterial infections<br>**2.** Tissue necrosis<br>**3.** Strenuous exercise<br>**4.** Convulsions<br>**5.** Tachycardia<br>**6.** Acute hemorrhage | |
| Hemoglobin | **1.** Polycythemia (increased in production of RBC) | **1.** Anemias<br>**2.** Lead Poisonings | Lymphocytes | **1.** Leukemia<br>**2.** Malnutrition<br>**3.** Viral infections | |
| Mean cell volume | **1.** Anemias<br>**2.** B-12 deficiency | **1.** Iron deficiency | Monocytes | **1.** Protozoal infections | |
| Mean corpuscular hemoglobin | **1.** Reticulocytosis | **1.** Iron deficiency | Eosinophils | **1.** Allergy<br>**2.** Irradiation<br>**3.** Pernicious anemia<br>**4.** Parasitism | |
| White blood cells | **1.** Bacterial infections<br>**2.** Bone marrow stimulation | **1.** Bone marrow depression<br>**2.** Cancer chemotherapy<br>**3.** Chemical intoxication<br>**4.** Splenic disorders | Basophils | **1.** Lead poisoning | |

also differences (though generally less striking) between different strains of rats and sometimes even between the animals supplied by difference sources.

### *Strain Differences*

Breeding rats for specific characteristics has produced some physiological differences between strains of rats. Some of these differences are known to affect how the various strains react to toxicants. Among others, strain specific differences have been found in sensitivity to thiourea (Dieke and Richter, 1945), sensitivity to acetaminophen nephrotoxicity (Newton et al., 1985a,b), the incidence of spontaneous glomerular sclerosis (Bolton et al., 1976), sensitivity to the carcinogenic actions of 7,12-dimethylbenz(a)anthracene (Boyland and Sydnor, 1962), the effects of trimethyltin on operant behavior and hippocampal glial fibrillary acidic protein (GFAP) (MacPhail et al., 2003), differences in renal

carcinogenesis (Hino et al., 2003), differences in cytochrome P4501A1 gene expression caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin in the liver (Jana et al., 1998), susceptibility to 4-nitroquinoline 1-oxide induce carcinoma (Kitano et al., 1992), and differences in the levels of drug-metabolizing enzymes (Page and Vesell, 1969). In recent years, research and breeding programs have been focused on producing inbred and outbred strains focused on specific disease models and susceptibility to the development of certain carcinomas. When choosing a strain for use, it is important to consider these differences.

Of importance for carcinogenicity studies, strain differences have been found in the incidence of spontaneous tumors. Table 2.5 gives the incidence of spontaneous tumors found in commonly used strains in carcinogenicity studies. The historical incidence is important to the analysis of a study in that a high spontaneous rate may mask a small test material—related increase in tumor incidence.

Because of lower spontaneous tumor rates, the Wistar has become the most popular strain in toxicological research.

### *Normal Physiological Values*

General values for selected physiological parameters are given in Tables 2.6 and 2.7. Normal values will vary based on the strain of animal, supplier, feed, and housing conditions. These tables should be used as a point of reference only.

## STUDY DESIGNS

The length and design of toxicology studies used to predict human risk are governed by guidelines issued by regulatory bodies such as the US Food and Drug Administration (FDA), the International Conference on Harmonization (ICH), the Environmental Protection Agency (EPA), and their counterparts worldwide. Toxicology studies are divided into a series of three sets of studies that are required for each phase of clinical trials. For initial approval to begin clinical trials, the following studies are required. The length of dosing in the toxicology studies varies depending on the intended length in clinical trials. A test compound intended to be a repeat dose study for up to 28 days in duration initially requires a two phase study in which a maximum tolerated dose (MTD) following a single administration is determined followed by a second phase during which the test compound is administered daily at dose levels based on the MTD for 5—7 days (Table 2.8). Following the completion of the MTD study a 14 or 28 Day Repeat Dose study should be conducted (Table 2.9). These studies assess the effects of a test compound at dosages that do not cause immediate toxic effects.

In support of Phase 2 clinical trials, longer-term subchronic and chronic toxicity studies (Table 2.10) should be conducted. Subchronic and chronic toxicity studies are designed to assess the test compound effects following prolonged periods of exposure. The highest dosage level in each of these studies should produce a toxic effect such that target organs may be identified. The lowest dosage level should provide a margin of safety that exceeds the human clinical dose and ideally allows for the definition of no observable effect level. Alternatively, when effects related to the pharmacological mechanism of the test compound or when observed effects may be related to treatment with the test compound but may not be of toxicologic significance, a no observable adverse effect level (NOAEL) may be determined.

In addition to the subchronic and chronic toxicity studies in support of Phase 2 clinical trials, reproductive

**TABLE 2.5** Incidence of Common Spontaneous Tumors in Fischer 344 and CD (SD)IGS Rats

| | | % Tumors in Untreated Rats | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CD(SD)IGS | | CD (SD) | | Fisher | | Wistar | |
| Organ | Tumor Type | Male | Female | Male | Female | Male | Female | Male | Female |
| Adrenal gland | Pheochromocytoma | 10.0 | 2.3 | 11.3 | 2.3 | 11.9 | 3.2 | 3.2 | 1.3 |
| Mammary gland | Fibroadenoma | 1.4 | 44.5 | 1.3 | 16.7 | 0.8 | 7.1 | 1.2 | 30.2 |
| Pancreas | Islet cell adenoma | 3.6 | 1.4 | 4.0 | 0.3 | 1.5 | 0.2 | 5.3 | 1.9 |
| Pituitary gland | Adenoma pars distalis | 33.6 | 56.8 | 35.7 | 50.3 | 12.4 | 28.2 | 41.1 | 65.8 |
| Testis | Interstitial cell tumor | 1.8 | | 7.0 | | 74.6 | | 4.3 | |
| Thyroid gland | C-Cell Adenoma | 10.5 | 5.0 | 5.0 | 5.7 | 12.5 | 8.2 | 10.1 | 10.7 |

*Adapted from Charles, R., 2011. Spontaneous Neoplastic Lesions in the Crl:CD BR Rat. Charles River Laboratories, Inc., Massachusetts; Mitsumori, K., Watanabe, T., Kashida, Y., 2001. Variability in the Incidence of Spontaneous Tumors in CD (SD) IGS, CD (SD), F244 and Wistar Hannover Rats in Biological Reference Data on CD(SD) IGS Rats, Yokohama, CD(SD) IGS Study Group.*

**TABLE 2.6** Selected Normative Data

| | |
|---|---|
| ***HUSBANDRY*** | |
| Room temperature (°C) | 18–26 |
| Relative humidity (%) | 30–70 |
| Ventilation (air change/h) | 10 |
| Light/dark cycle (h) | 12–14/12–10 |
| Minimum cage floor size | |
| Housed individually ($cm^2$) | 350 |
| Breeding with pup ($cm^2$) | 800 |
| Group housed ($cm^2$ adult) | 250 |
| ***GENERAL*** | |
| Life span (years) | 2.5–3.0 |
| Surface area ($cm^2$) | 0.03–0.06 |
| Chromosome number (diploid) | 42 |
| Water consumption (mL/100 g/day) | 10–12 |
| Food consumption (g/day) | 20–40 |
| Average body temperature (°C) | 37.5 |
| ***REPRODUCTION*** | |
| Puberty (males and females) | 50 ± 10 days |
| Breeding season | All year |
| Type of estrous cycle | Polyestrous |
| Length of estrous cycle | 4–5 days |
| Duration of estrous | 10–20 h |
| Mechanism of ovulation | Spontaneous |
| Time of ovulation | 7–10 h after onset of estrous |
| Time of implantation | Late day 4 or 5[a] |
| Length of gestation | 21–23 days |
| Litter size | 8–16 pups |
| Birth weight | 5–6 g |
| Eyes open | 10–12 days |
| Weaning age/weight | 21 days/40–50 g |
| ***CARDIOVASCULAR*** | |
| Arterial blood pressure | |
| Systolic (mmHg) | 116–145 |
| Diastolic (mmHg) | 76–97 |
| Heart rate (beats/min) | 296–388 |
| Cardiac output (mL/min) | 10–80 |
| Blood volume (mL/kg) | 64 |
| ***PULMONARY*** | |
| Respiration (breaths/min) | 100–140 |
| Tidal volume (mL) | 1.1–2.5 |
| Compliance (mL/cm $H_2O$) | 0.3–0.9 |
| Resistance (cm $H_2O$/mL s) | 0.1–0.55 |
| Pattern | Obligate nasal |
| ***RENAL*** | |
| Urine volume | 15–30 mL/24 h |
| $Na^+$ excretion | 200 mmol/L/24 h |
| $K^+$ excretion | 150 mmol/L/24 h |
| Urine osmolarity | 2000 mOsm/kg $H_2O$ |
| Urine pH | 7.3–8.5 |
| Urine specific gravity | 1.01–1.07 |
| Urine creatinine | 6 μmol/L/24 h |
| Glomerular filtration rate | 1.0 mL/min/100 g body weight |

[a]*The estrous cycle length may vary from 4 to 5 days between strains. Time of implantation may vary based upon the length of the estrous cycle and is dependent upon Day 0 or the first day sperm is found in the vagina.*

*Data from Baker, H.J., Lindsey, J.R., Weisbroth, S.H., 1979. Housing to control research variables. In: Baker, H.J., Lindsey, J.R., Weisbroth, S.H. (Eds.), The Laboratory Rat, vol. 1. Academic Press, New York, pp. 169–192; Hofsteller, J., Svekow, M.A., Hickman, D.L., 2006. Morphophysiology. In: Svekow, M.A., Weisbroth, S.H., Franlin, C.L. (Eds.), The Laboratory Rat, second ed. vol. I. Academic Press, New York, pp. 93–125; Peplow, A., Peplow, P., Hafez, E., 1974. Parameters of reproduction. In: Vo, I., Melby, E., Altmon, N. (Eds.), Handbook of Laboratory Animal Science. CRC Press, Boca Raton, pp. 107–116; Waynforth, H., Flecknell, P., 1980. Experimental and Surgical Technique in the Rat, second ed. Elsevier Academic Press; Sharp, P., LaRegina, M., 1998. The Laboratory Rat. Academic Press, Philadelphia; Van Zutphen, L.F.M., Baumans, V., Beynen, A.C., 1993. Principles of Laboratory Animal Science. Elsevier, Amsterdam.*

safety studies may also be required. Reproductive toxicity studies are typically required for a test compound intended to be administered to women of childbearing age or may affect male reproduction. These studies include an assessment of the potential effects of the test compound on general fertility and reproductive performance (Segment I), developmental toxicity (Segment II), or affect perinatal and postnatal development (Segment III). The highest dose in reproductive studies should be chosen so that administration causes some minimal toxicity. Typically, a dose range finding pilot study in a small number of animals should be conducted prior to initiating the definitive reproductive toxicology studies. Examples of protocols designed to meet the ICH guidelines are presented in Tables 2.9, 2.9A, and 2.10.

In support of Phase 3 clinical trials, two carcinogenicity studies may be required (Table 2.8), one in rats and one in mice. Typically 18 months to 2 years in duration, this type of study is designed to assess the potential of the test compound to induce neoplastic lesions. The highest dosage in a carcinogenicity study should cause minimal toxicity when administered via the intended route for clinical use. The preclinical studies required

**TABLE 2.7** Growth Rates in Selected Rat Strains

| | Age (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Crl:CD (SD)IGSBR | | Crl:(WI)BR | | Crl:(LE)BR | | CDF(F-344)/CrlBR | |
| **Weight (g)** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| **Up to 50** | **Up to 23** | **Up to 23** | **Up to 23** | **Up to 25** | **Up to 21** | **Up to 21** | **Up to 23** | **Up to 23** |
| 51–75 | 24–28 | 24–29 | 24–28 | 26–30 | 22–25 | 22–26 | 24–29 | 24–29 |
| 76–100 | 29–34 | 30–35 | 29–32 | 31–34 | 26–29 | 27–31 | 30–34 | 30–35 |
| 101–125 | 35–37 | 36–39 | 33–35 | 35–40 | 30–34 | 32–36 | 35–39 | 36–42 |
| 126–150 | 38–42 | 40–44 | 36–40 | 41–47 | 35–37 | 37–43 | 40–45 | 43–55 |
| 151–175 | 43–45 | 45–50 | 41–44 | 48–56 | 38–42 | 44–50 | 46–50 | 56–72 |
| 176–200 | 46–49 | 51–56 | 45–48 | 57–64 | 43–46 | 51–55 | 51–57 | 73–105 |
| 201–225 | 50–52 | 57–70 | 49–52 | 65–81 | 47–49 | 56–69 | 58–63 | 105+ |
| 226–250 | 53–56 | 71–84 | 53–56 | 82–105 | 50–55 | 70–86 | 64+ | |
| 251–275 | 57–59 | 84–105 | 57–61 | 106+ | 56–58 | 87–102 | | |
| 276–300 | 60–65 | 106+ | 62–67 | | 59–64 | 103+ | | |
| 301–325 | 66–71 | | 68–73 | | 65–70 | | | |
| 326–350 | 72–77 | | 74–79 | | 71–80 | | | |
| 351–375 | 78–87 | | 80–87 | | 81–90 | | | |
| 376+ | 88+ | | 88+ | | 91+ | | | |

*Adapted from Charles, R., 2004. Growth Rates in Selected Rat Strains. Charles River Laboratories, Inc., Massachusetts.*

in support of the clinical trials are dependent on the intended route and frequency of administration of the test compound and the intended age group to be treated (Tables 2.11–2.14).

## ROUTES OF TEST ARTICLE ADMINISTRATION

### Oral Routes

Rodents have several unique characteristics to be considered regarding the oral administration of test compounds. One of the most important characteristics is the lack of an emetic response. The lack of this response allows for a higher dose of a potential emetic compound to be administered and evaluated. Many compound and excipients may cause emesis in dogs or other large animal species and may lead to a low level of exposure and erratic blood levels. A second factor to consider is that rodents are nocturnal and eat most of their food at night. When maintained on a 12 h light–dark cycle, rats have been found to consume 75% of their daily food intake during the dark cycle (Wong and Oace, 1981). This should be taken into consideration when designing an oral gavage study and determining when the animal may be dosed. Early in the light cycle, animals are more likely to have a full stomach and complications associated with dosing may occur if large volumes of test article are administered. In addition, a full stomach may affect gastric emptying and the rate of absorption of an orally administered test compound.

Techniques for oral administration of test compounds include mixing in the diet, via gavage or stomach tube, via capsule, or in drinking water. The most widely used methods of oral administration are the dietary and gavage techniques.

#### *Dietary Versus Gavage Methods*

The choice between dietary and gavage dosing techniques is typically based on several factors. A scientific decision can only be made with a knowledge of the pharmacokinetics of the test compound administered by both methods. Other considerations that may be used in making this decision are as follows.

The dietary method can be used if a compound can be mixed with the diet, is stable under storage conditions in the diet, and is palatable to the animal. A major advantage of the dietary method is that it requires less manpower to perform the study. The diet mixing process can be performed weekly or, if stability allows, less often. The mixing and feeding process is less labor-intensive than gavaging rats on a daily basis.

**TABLE 2.8** Maximum Tolerated Dose Study in Rats

| **Phase A** | **Oral MTD Study** | |
|---|---|---|
| | **Males** | **Females** |
| Dose Level 1 | 3 | 3 |
| Dose Level 2 | 3 | 3 |
| Dose Level 3 | 3 | 3 |
| Dose Level 4 | 3 | 3 |

| **Phase B** | **7-Day Oral Range Finding Study** | | | |
|---|---|---|---|---|
| | **Main Study** | | **Toxicokinetics** | |
| | **Males** | **Females** | **Males** | **Females** |
| Control | 5 | 5 | – | – |
| Low dose | 5 | 5 | 9 | 9 |
| Mid dose | 5 | 5 | 9 | 9 |
| High dose | 5 | 5 | 9 | 9 |

***Experimental Design***:
*In Phase A, the dose level will be increased until the maximum tolerated dose (MTD) is determined. The MTD is a dose that produces neither mortality nor more than a 10% decrement in body weight nor clinical signs of toxicity. In Phase B, animals will be dosed daily for 7 days at fractions of the single dose MTD to estimate a repeat dose MTD.*
***Dose Route/Frequency***:
*As requested.*
*Phase A: Once.*
*Phase B: Once per day for 7 consecutive days.*
***Observations***: *Twice daily in both phases (mortality/moribundity).*
***Detailed Clinical Observations***: *Daily in both phases.*
***Body Weights***: *Daily in both phases.*
***Food Consumption***: *Daily.*
***Clinical Pathology (Phase B only)***: *Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination.*
***Necropsy (Phase B only)***: *Tissues saved for possible future histopathological evaluation.*
***Organ Weights (Phase B only)***: *Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus.*
***Toxicokinetics***: *Blood collected on days 1 and 7 (three cohorts consisting of three animals/sex/treatment group bled twice to equal six time points), calculation of* $C_{max}$, $T_{max}$, $AUC_{0\text{-}24}$, *and* $T_{1/2}$.

Several disadvantages also exist in using the dietary method. Methods must be developed and validated to prove homogeneity and stability. This is not as easy a process as with a suspension or solution. The dietary method is also less exact than the gavage method, in that the concentration of compound mixed in the feed is based on predicted feed consumption and body weights. In addition, if the feed is not palatable to the animal, or the test compound makes the animal ill, feed consumption may be reduced thereby reducing exposure to the test compound. In addition, the facility and control animals may be exposed to the test compound through dust or vapors.

**TABLE 2.9** 14 or 28 Day Repeat Dose Toxicity Study in Rats

| | **Main Study*** | | **Toxicokinetics** | |
|---|---|---|---|---|
| | **Males** | **Females** | **Males** | **Females** |
| Vehicle control | 10 | 10 | – | – |
| Low dose | 10 | 10 | 9 + 3[a] | 9 + 3[a] |
| Mid dose | 10 | 10 | 9 + 3[a] | 9 + 3[a] |
| High dose | 10 | 10 | 9 + 3[a] | 9 + 3[a] |

***Observations***: Twice daily (mortality/moribundity).
***Detailed Clinical Observation***: *Weekly.*
***Functional Observational Battery***: *Pretest and Day 14 or 25.*
***Body Weights***: *Weekly.*
***Food Consumption***: *Weekly.*
***Ophthalmology***: *All animals prior to test article administration; all surviving main study animals at study termination.*
***Clinical Pathology***: *Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination.*
***Toxicokinetics***: *Blood collected on Days 1 and 14 or 27 (three cohorts consisting of three animals/sex/treatment group bled twice to equal six time points); TK modeling. The use of subsets of all the animals in a test group is called "spare sampling," intended to avoid the need for additional ("Satellite") groups of animals. Note that although six time points are commonly collected, more may be required or taken to adequately characterize a drug's pharmacokinetics.*
***Necropsy***: *All main study animals; toxicokinetic animals euthanized and discarded.*
***Organ Weights***: *Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus.*
***Slide Preparation/Microscopic Pathology***: *All animals in the vehicle control and high dose groups and all found dead animals: full set of standard tissues; low and mid dose group target organs (to be determined); gross lesions from all animals.*
[a]*Three additional animals/sex/treatment group included as replacement animals.*
** Should also refer to table note "a".*

The gavage method may be used when the test compound is not stable in the diet or may not be palatable to the animals. In addition, the gavage method is preferable when evaluating toxicokinetics or pharmacokinetics. As with dietary mixtures, test compound administered via gavage as a solution or suspension should be analyzed for homogeneity, stability, and concentration. Methods for solution or suspension may be easier to develop than those required for dietary mixtures. For Good Laboratory Practices (GLP) studies, evaluation of homogeneity, stability, and concentration should be conducted for every study. If the same methodology and batch size are used for multiple studies, homogeneity may be established once. Stability of the test compound in solution or suspension should be determined under the testing conditions in the proposed vehicle. Typically, stability for toxicology studies is established for between 7 and 14 days. If the test compound is not found to be stable, stability of shorter duration may be established. Lastly, concentration analysis should be established for each dose level and should be periodically evaluated during longer-term studies.

With the gavage method of dosing, a more precise amount of the test compound can be delivered and

**TABLE 2.9A** 28 Day Repeat Dose Toxicity Study With Immunophenotyping in Rats

| | Main Study | | Toxicokinetics | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| Vehicle control | 10 | 10 | | |
| Low dose | 10 | 10 | 9 + 3[a] | 9 + 3[a] |
| Mid dose | 10 | 10 | 9 + 3[a] | 9 + 3[a] |
| High dose | 10 | 10 | 9 + 3[a] | 9 + 3[a] |

***Observations***: Twice daily (mortality/moribundity).
***Detailed Clinical Observation***: *Weekly*.
***Functional Observational Battery***: *Pretest and Day 25*.
***Body Weights***: *Weekly*.
***Food consumption***: *Weekly*.
***Ophthalmology***: *All animals prior to test article administration; all surviving main study animals at study termination*.
***Clinical Pathology***: *Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination*.
***Immunotoxicology***: *Immunophenotyping of blood leukocytes by flow cytometry on all surviving main study animals at termination. NK cell assay on blood leukocytes of all surviving main study animals at termination. May include identification of any antidrug antibodies (ADAs)*.
***Toxicokinetics***: *Blood collected on Days 1 and 27 (three cohorts consisting of three animals/sex/treatment group bled twice to equal six time points)*.
***Necropsy***: *All main study animals; toxicokinetic animals euthanized and discarded*.
***Organ Weights***: *Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate,* ***salivary*** *glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus, two lymph nodes (e.g., mesenteric, axillary, popliteal, etc.) including the lymph node draining the route of administration*.
***Slide Preparation/Microscopic Pathology***: *All animals in the vehicle control and high dose groups and all found dead animals: full set of standard tissues (add Peyer's patch, extra lymph node); low and mid dose group target organs; gross lesions from all animals*.
[a]*Three additional animals/sex/treatment groups included as replacement animals; the control animals will not be evaluated for toxicokinetics.*

**TABLE 2.10** Subchronic and Chronic Toxicity Study in Rats

| | Main Study | | Toxicokinetics | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| Vehicle control | 15 | 15 | – | – |
| Low dose | 15 | 15 | 9+3[a] | 9+3[a] |
| Mid dose | 15 | 15 | 9+3[a] | 9+3[a] |
| High dose | 15 | 15 | 9+3[a] | 9+3[a] |

***Observations***: Twice daily (mortality/moribundity).
***Detailed Clinical Observation***: *Weekly*.
***Body Weights***: *Weekly*.
***Food Consumption***: *Weekly*.
***Ophthalmology***: *All animals prior to test article administration; all surviving main study animals at study termination*.
***Clinical Pathology***: *Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination*.
***Toxicokinetics***: *Blood collected on Days 1 and 90 (three cohorts consisting of three animals/sex/treatment group bled twice to equal six time points); TK modeling*.
***Necropsy***: *All main study animals; toxicokinetic animals euthanized and discarded*.
***Organ Weights***: *Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus*.
***Slide Preparation/Microscopic Pathology***: *All animals in the vehicle control and high dose groups and all found dead animals: full set of standard tissues; low and mid dose group target organs; gross lesions from all animals*.
[a]*Three additional animals/sex/treatment group included as replacement animals.*

may reduce the amount of test compound required to complete the study. This becomes important when evaluating the effects of a pharmaceutical, as the required dose levels and exposure levels to show safety may be lower than that required for a pesticide or chemical. A disadvantage of the gavage method is that it involves handling of the rat for each dosing. Handling of the rat has been shown to increase corticosterone levels (Barrett and Stockham, 1963) and may affect study results. Additionally, daily intubation may lead to death due to esophageal puncture or inhalation pneumonia.

### *Dietary Method*

When utilizing the dietary method, the test compound is mixed with the diet and administered to the animals either ad libitum or the diet is presented to the animals for a fixed amount of time each day. The dosage received by an animal is regulated by varying the concentration of test compound in the diet based on the predicted food consumption and body weight. Food consumption and body weight predictions are based on historical laboratory data for early time points in a study. As the study progresses, growth and food consumption curves can be established for each group

**TABLE 2.11** Study of Fertility and Early Embryonic Development to Implantation in Rats

| | Males | Females |
|---|---|---|
| Vehicle control | 25 | 25 |
| Low dose | 25 | 25 |
| Mid dose | 25 | 25 |
| High dose | 25 | 25 |

***Dose Route/Frequency***: Males dosed began 28 days before mating and continued until euthanasia. Females dosed began 14 days before mating and continued through Day 7 of gestation (implantation).
***Observations***: *Twice daily (mortality/moribundity)*.
***Clinical Examinations***: *Observations for clinical signs, body weights, and food consumption measurements recorded during the study period. Beginning at initiation of test article administration, females examined daily to establish estrous cycle*.
***Uterine Examinations***: *Performed on dams on Day 13 of gestation. Gravid uterine weight and the weight of the ovaries recorded. Total number of corpora lutea and implantations, location of resorptions, and embryos recorded. Females subjected to necropsy, and reproductive organs and gross lesions fixed for possible microscopic evaluation*.
***Evaluation of Males***: *Following disposition of females, the males were euthanized and subjected to a necropsy. The testes and epididymides weighed, and analysis of sperm parameters (concentration, motility, and morphology) performed. Reproductive organs and gross lesions fixed for possible microscopic evaluation*.
***Statistical Analysis***: *Standard*.

**TABLE 2.12** Embryo-Fetal Development in Rats

| | Time Mated Females |
|---|---|
| Vehicle control | 25 |
| Low dose | 25 |
| Mid dose | 25 |
| High dose | 25 |

***Dose Route/Frequency***: Dosing initiated on Day 6 of gestation and continued to include Day 17 of gestation.
***Observations***: *Twice daily (mortality/moribundity).*
***Clinical Examinations***: *Daily Gestation Days 6 through 20.*
***Body weights/Food Consumption***: *Gestation Days 0, 6, 9, 12, 15, 18, and 20.*
***Cesarean Section/Necropsy***: *Litters will be delivered by cesarean section on Day 20 of gestation. Gravid uterine weight will be recorded. Total number of corpora lutea, implantations, early and late resorptions, live and dead fetuses, and sex and individual body weights of fetuses will be recorded. External abnormalities of fetuses will be recorded. Approximately one-half of the fetuses will be processed for visceral abnormalities, and the remaining fetuses will be processed for skeletal abnormalities. Dams will be subjected to a necropsy and gross lesions and target organs (if known) will be saved.*

**TABLE 2.14** Carcinogenicity Study in Rats

| | Main Study | | 6-Month Satellite | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| Vehicle control | 60 | 60 | 20 | 20 |
| Low dose | 60 | 60 | 20 | 20 |
| Mid dose | 60 | 60 | 20 | 20 |
| High dose | 60 | 60 | 20 | 20 |

***Study Desgin***: Group as per Table 2.14.
***Observations***: *Twice daily (mortality/moribundity).*
***Detailed Clinical Observations***: *Once weekly.*
***Body Weights***: *Weekly for first 13 weeks, monthly thereafter.*
***Food Consumption***: *Weekly for first 13 weeks, monthly thereafter.*
***Ophthalmology***: *All animals pretest and all survivors prior to terminal sacrifice.*
***Clinical Pathology***:
*Main Study: Hematology at termination.*
*6-Month Satellite: Hematology, clinical chemistry, and urinalysis evaluations on all surviving satellite animals at termination.*
***Necropsy***: *All animals.*
***Slide Preparation/Microscopic Pathology***: *All animals, full set of standard tissues, all masses, and all lesions.*
***Statistical Analysis***: *Standard.*

and group mean data can be used to predict future food consumption. Different concentrations of the test compound and diet should be made for each sex.

Test compounds and diets are mixed in two steps: (1) the compound and about 10% of the total amount of diet are blended in a premix, then (2) the premix and the remainders of the diet are mixed. The total amount of diet to be mixed is first weighed out, the 10% is separated into the premix. To make the premix, the entire test compound and an aliquot of the diet (from the 10%) are put into a mortar. These ingredients are ground with a pestle

**TABLE 2.13** Pre- and Postnatal Development, Including Maternal Function in Rats

| | P Generation (F0) | | F1 Generation | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| Vehicle control | NA | 25 | 25 | 25 |
| Low dose | NA | 25 | 25 | 25 |
| Mid dose | NA | 25 | 25 | 25 |
| High dose | NA | 25 | 25 | 25 |

***Number in Study***: P Generation—100 females, F1 Generation—100 males, 100 females.
***Dose Route/frequency***: *Once daily to P animals from Gestation Day (GD) 6 to Postnatal Day (PND) 21. F1 animals not dosed.*
***Observations***: *Twice daily (mortality/moribundity).*
***Clinical Observations***: *P females—daily during treatment/F1 adults—weekly.*
***Body Weights***:
*P females—GD 0, 6, 10, 14, 17, and 20, PND 0, 7, 10, 14, and 21.*
*F1 males—Weekly through termination.*
*F1 females—Weekly until evidence of copulation detected, then GD 0, 7, 10, and 13.*
***Food Consumption***: *P females—On corresponding body weight days during gestation/lactation.*
***Vaginal Smears***: *All F1 females during a 21-day cohabitation period until evidence of copulation is detected.*
***Litter Evaluations***: *All F1 offspring, count, body weight, sex, clinical observations on PND 0, 4, 7, 14, 21; behavioral and developmental evaluation of four males and four females from each litter for static righting, pinna detachment, cliff aversion, eye opening, air drop righting reflex, neuropharmacological evaluation, auditory response. One male and one female (selected for the next generation) tested for sexual maturation (vaginal opening, preputial separation), motor activity/emotionality, and passive avoidance.*
***Cesarean Section***: *On GD 13, F1 females for location of viable and nonviable embryos, early and late resorptions, number of total implantations, and corpora lutea.*
***Sperm Evaluation***: *May be conducted on F1 males if evidence of reduced fertility is noted (additional cost).*
***Necropsy***: *Gross lesions/target organs fixed for possible microscopic evaluation (additional cost).*
*All P females at PND 22 as well as all F1 weanlings not selected for F1 generation.*
*All F1 females at GD 13.*
*All F1 males after termination of F1 cesarean sections.*

until the mixture appears homogeneous. The mixture and the remainder of the premix are then layered in a small capacity mixer and mixed for 5–10 min. The time for this mixing process can be varied if analysis shows the total mixture is not homogeneous. For the final mix, the premix and the remainder of the diet are layered in a large capacity mixer. The mixing time will vary with the type of blender and can be varied if the analysis shows the total mixture is not homogeneous.

Several types of blenders are available for the mixing process; these include open-bowl "kitchen" mixers, V or PK blenders, and Turbula mixers. Metal parts should be ground to eliminate electrostatic forces. In addition, alternative methods of dietary administration such as microencapsulation may be used for volatile, reactive, or unpalatable chemicals.

### *Gavage Method*

In the gavage procedure, the test compound is administered by passing a feeding tube or gavage needle attached to a syringe down the esophagus into the stomach.

#### Test Article Preparation

If not already a liquid, the test compound is prepared for administration by adding it to the appropriate vehicle. The choice of vehicle will depend on the characteristics of the compound and whether it is to be administered as a suspension or a solution. In addition, consideration must be given to the effects of the vehicle on the rat (Gad and Chengelis, 1998). Common vehicles used include water and food grade oils such as corn oil. Suspensions are made when aqueous vehicles are desired and the test compound is not soluble. Suspending agents such as methylcellulose are added to increase the viscosity and hold the compound in suspension. Other agents such as Tween 80, ethanol, polyethyleneglycol 400 (PEG 400), and others may be used as wetting or stabilizing agents.

#### Equipment

Soft catheters made of silastic or polyethylene (e.g., infant feeding tubes), stainless steel gavage needles with smooth ball-shaped tips, or polyethylene gavage needles with ball-shaped tips are commonly used. All are commercially available and are relatively inexpensive. Although the soft catheter minimizes the chance of esophageal trauma, liquid can leak past the catheter and back up the esophagus and be aspirated. The ball-shaped tips of the stainless steel gavage needles reduce the chances of tracheal injections; however, if an animal struggles while the needle is in the esophagus, the rigid needle increases the chances of perforating the esophagus. The polyethylene gavage needle incorporates the best of both the soft catheter and the stainless steel needle, but because of the flexible nature of the needle, the risk for tracheal injection is increased.

Conybeare and Leslie (1980) found that deaths in gavage studies were a result of aspiration of small amounts of irritant solutions or acidic, hypertonic solutions. They also found that the use of a ball-tip 4 mm in diameter helped to eliminate deaths related to dosing. With gentle handling, the animals will be acclimated to the techniques used and dosing will become easier.

Aspiration and tracheal administration of test compound as well as esophageal trauma have been associated with gavage dosing and may lead to difficulty in interpretation of the study. The catheter and the needles all have risks inherent in their use; therefore, care should be taken when using these tools and animal technicians should be properly trained. The choice of the appropriate catheter or needle should be left up to the technician and should be whatever the technician has been trained and is most comfortable with.

#### Technique

The description below is appropriate for either a gavage needle or catheter; for simplicity, only the needle will be mentioned in the description. Prior to picking up the animal, the syringe should be attached to the needle and filled with the appropriate amount of test compound to be delivered. Any air bubbles should be eliminated and the needle wiped clean of residual test compound. This is done so that the animal does not taste the test compound and residual test compound is not aspirated as the needle is passed down the esophagus. If the dosing liquid is distasteful, the animal may struggle after repeated dosing and increase the chances of being injured.

To position the animals for gavage, it should be grasped by the skin of the back and neck ensuring that the head, neck, and back are in a straight line. Alternatively, the animals can be grasped about the shoulders, with the index finger and thumb on either side of the head. The objective is to firmly hold the animals to be able to control any struggling if it occurs and to also prevent the animal from being able to bite the technician. For even more control, the animal may be placed on a table or brought up against the operator's chest.

Once the animal is in position, the needle can be inserted into the mouth of the animal, moved over the tongue, and down into the esophagus. The length of the needle should be inserted into the animal. A slight rotation of the needle may help with insertion into the esophagus. If the needle is inserted into the trachea, the animal may struggle. The syringe should be grasped lightly such that, if the animal does struggle, the chances of an esophageal tear are minimized. If the animal continues to struggle, the needle should be withdrawn to allow the animal to calm down, and then dosing should

be attempted again. Alternatively, if a catheter is used, as the tube is placed into the mouth, it should be placed to the side between the molars. This is done because the tube may by bitten or transected if passed too close to the front teeth.

With the needle in place, the test compound should be slowly expelled into the animal. If administered to rapidly, reflux may occur and the test compound may back up into the esophagus, resulting in an inaccurate dose being given and possible aspiration of the test compound. Once the dose has been delivered, the needle should be withdrawn and the animal observed for any signs of distress or respiratory difficulty. An experienced technician should be able to dose between five and seven animals per minute without causing discomfort to the animals and with minimal dosing-related deaths.

Gavage liquids are commonly administered at a volume of 5–10 mL/kg body weight. The volume should be enough to be delivered accurately, but not so much that it will adversely affect the animal. The maximum volume should be no more than 20 mL/kg. If using volumes greater than 10 mL/kg, it may be advisable to fast the animals for several hours prior to dosing. This will ensure that the stomach is empty prior to dosing and able to handle the larger volume. This option should be considered carefully, as fasting can affect the rate of absorption and clearance from the stomach. In addition, the choice of housing and bedding should be considered when dosing with large volume as rats have the tendency to eat the bedding, which may hinder gavage dosing. In addition, the volume chosen can have an effect on the results of the study and volumes greater the 10 mL/kg should only be used when issues of solubility and exposure exist. Ferguson (1962) found that a change in dose volume of from 5% to 1% of body weight could reduce mortality rate from approximately 95%–5%, respectively, at equivalent doses.

### Neonatal Administration

Neonatal intragastric injections can be made orally with thin silicone tubing (Gibson and Becker, 1967; Smith and Kelleher, 1973) or by intragastric injection with a 27-gauge needle through the abdominal wall (Worth et al., 1963; Bader and Klinger, 1974). The oral method using silicone tubing is performed in a similar manner to the previously described method in adult rats. The intragastric injection through the abdominal wall is performed by first locating the stomach in the upper left quadrant of the abdomen and then carefully inserting the needle through the abdominal wall into the stomach taking care that the animal does not move. The syringe should be gently aspirated to ensure proper placement and then the injection completed and the needle withdrawn.

### Capsule

To eliminate the possibility of dosing errors and to deal with compounds that cannot be delivered through conventional means, methods have been developed for the administration of capsules into the esophagus of the rat. The test compound may be prefabricated into a small capsule or the test article may be weighed and placed into commercially available capsules. An individual capsule is then placed into a specially designed cup in the end of a gavage needle, and the needle is then inserted into the esophagus of the rat. The capsule is then pushed out of the cup into the esophagus using either air or a rod inside the needle. The needle is then withdrawn and the capsule moves down into the stomach by peristaltic action. Only a small amount of test compound can be administered as a single dose using this method, but multiple capsules can be administered sequentially in the same dosing session.

### Water

As an alternative to dietary administration, compounds that are water soluble, palatable to the rat, and stable in water may be administered via the drinking water. This method offers similar advantages as adding a test compound to the diet. Additionally, compounds will be more easily mixed and analyses will be more easily developed than when a compound is in the diet. However, spillage of water makes measurement of the actual dose received difficult.

### Intravenous Route

One of the most common methods of administration of test compound is via intravenous (iv) injection or infusion. The iv route is often the route of choice for compounds that have poor bioavailability via the oral route or have a short half-life. Several issues must be considered when administering a test compound intravenously. The compound must be soluble in an acceptable iv vehicle or excipient, must be able to be administered as a solution, and should be sterile or sterile filtered prior to administration. In addition, when designing a study, the pharmacokinetic profile of the test compound administered intravenously should be considered. Study activities such as clinical observations and functional observational battery should be planned around the expected time of greatest plasma concentration.

A variety of veins may be used for iv injections (Diehl et al., 2001). These include the lateral tail (caudal), jugular, femoral, saphenous, lateral marginal, dorsal metatarsal, sublingual, and dorsal penile vein. Although most of these are superficial, and easily available for injection, several require the use of anesthesia or more than one technician and may be of limited use in repeat

dose studies. Although anesthesia may be acceptable for acute studies or surgical model, its repeated use may have an effect on the toxicity of a test compound.

### *Lateral Tail Vein*

The lateral tail veins are currently the most widely used for iv injections in the rat. The veins are easily visible, especially in young animals and injections can be performed by one person without the use of anesthesia. The technician performing the function should be well trained and care should be taken to ensure that the lateral veins are being accessed and not the dorsal or ventral artery of the vein.

#### Bolus Injection

The animal should be placed in an appropriate restrainer. This typically consists of a solid tube in which the animal is placed into headfirst and has a stop that is placed behind the animal with a hole that allows the tail to hang out the back. The restraint tube is designed to be secure enough that the animal cannot move, back out, or turn, but can still breathe comfortably. Once secure, the tail should be cleaned and the vein may be dilated with heat. This may be accomplished by placing the tail in warm water (40–45°C), placed under a heat lamp, or wrapped with warm gauze. Care must be taken to avoid using excessive heat as tissue damage may result. Minasian (1980) describes a tourniquet made from a plastic syringe and thread. If used, this should not be left on for an extended period of time.

When performing an injection, the end of the tail should be held firmly and taut with the thumb and index finger of one hand. A 23-gauge needle attached to an appropriately sized syringe should be held with the bevel up at a shallow angle parallel to the vein. The skin of the tail is then pierced and the needle advanced until resistance is no longer felt. The plunger of the syringe should then be aspirated to ensure proper placement of the needle. The use of a needle with a clear or transparent hub will facilitate confirmation of correct placement. Blood backflow into the needle confirms entry into the vein. Alternatively, a butterfly needle with an extension line may be used. The butterfly needle with an extension set precludes the need to hold the tail, needle, and syringe. When using this type of setup, the butterfly needle is attached to an extension set and syringe that is filled with the test compound. The tail may be taped to the table, and the butterfly needle is then inserted into the vein and placement is verified by aspiration on the syringe. Once confirmed, the butterfly needle may also be taped in place. This prevents the needle from pulling out of the vein during dosing. This type of setup can be very useful when administering large volumes of test article as a slow bolus over several minutes or when the test compound may be irritating or mildly caustic. Taping the animal's tail in place prevents the animal from pulling the tail out of the fingers of the technician.

If repeated dosing is to be performed, the initial venipunctures should be performed as close to the tip of the tail as possible. During the injection, if the needle comes out of the vein, a bleb will form under the skin. The needle should be repositioned immediately to prevent infiltration of the solution around the vein. Infiltration of an irritating solution can cause necrosis and make future injections difficult or impossible. Injection of 2 mL/100 g body weight can be accomplished without stress to the rat. Barrow (1968) found that injections of volumes over this amount produced respiratory difficulty and pulmonary edema.

### *Tail Vein Infusions*

Tail vein infusions are convenient because catheter placement can be accomplished without anesthesia. A 23-gauge or smaller needle connected to an extension set is inserted into the tail. The needle and extension set is then secured to the tail with tape. The extension set is attached to a syringe that is placed on a pump and the test compound can be infused. The tail may be taped to a wooden stick or tongue depressor to further protect the needle from being dislodged. Over the needle, catheters are also commercially available and offer the advantage that the needle is removed once the catheter is placed in the vein and may help to prevent further penetration of the vein wall and subsequent perivascular dosing (Rhodes and Patterson, 1979). Advantages that this technique has over permanent indwelling catheters are that the catheter is removed following dosing and will not become occluded and the animal doses not have to undergo anesthesia and a surgical procedure to place the catheter. Permanent catheters have a tendency over time to develop a fibrin flap or become clotted, thus losing patency. A major disadvantage is that the animals have to be restrained during the infusion, which may causes stress and alter the results of the study. When using this technique, the duration of the infusion should be limited so that the length of time the animal is restrained is limited.

An alternative technique using the lateral tail vein involves placing a catheter in the vein and wrapping the tail in a similar manner as previously described, then a lightweight protective cover attached to a tether system is placed around the tail to hold the catheter or needle in place.

### *Jugular Vein*

Although this route has been used for bolus injections, it is most widely used as a site for cannulation from indwelling catheters. The indwelling catheter requires surgical implantation under anesthesia.

### Bolus Injection

Although injections can be made by exposing the jugular vein by incision, this method is not acceptable for repeated dosing. The jugular vein can be accessed for test compound administration without exposing the vein. The animal can either be anesthetized or restrained on the back. The head is positioned to either the left or the right for access to the respective jugular vein. A 23-gauge needle fitted to a syringe with the bevel up is inserted in a cephalocaudal direction into the angle made by the neck and shoulder. The needle should enter the vein anterior to the point at which it passes between the pectoralis muscle and the clavicle. When about one-half the length of the needle has penetrated the skin, the bevel should be in the lumen of the vessel. Insertion of the needle through the muscle stabilizes the needle and minimizes bleeding. Caution should be used when using this technique as it is considered to be a "blind stick" into the vessel, and damage to the vessel may occur. Repeated access of the vessel is not recommended.

### Infusion

For the purpose of continuous infusion of the test compound over extended periods of time or for repeated short-term infusions, implanted catheters in the jugular vein may be used. For implantation of a jugular catheter, the animal is first anesthetized and placed in dorsal recumbency, and the surgical site is prepared. A midline incision is then made in the neck, and a section of the jugular vein is dissected free. Manipulation of the vein should be limited to prevent vasospasm. A cephalic ligature is then tied and the vein elevated. A small incision is then made in the vein, and the catheter is passed into the vein and tied in place. The other end of the catheter is then tunneled subcutaneously (sc) to between the scapula where the catheter is exteriorized. The catheter should be filled with an anticoagulant solution such as heparin when not in use. When correctly positioned, the tip of the cannula will be at the junction of both vena cava. If placing catheters into young animals, enough of the catheter should be inserted to allow for growth of the animal. Care should be taken that the catheter is not inserted too far as the tip may be pushed into the right ventricle of the heart. Improper placement of the catheter may lead to administration of the test compound directly into the heart, which can cause complications.

Similar to the jugular vein, administration of test article via the femoral vein requires an implanted catheter. For implantation of a femoral catheter, the animal is first anesthetized and placed in dorsal recumbency, and the surgical site is prepared. A midline incision is then made in the inguinal area and a section of the femoral vein is dissected free. Manipulation of the vein should be limited to prevent vasospasm. A ligature is then tied and the vein elevated. A small incision is then made in the vein and the catheter is passed into the vein and tied in place. The other end of the catheter is then tunneled sc to between the scapula where the catheter is exteriorized. The catheter should be filled with an anticoagulant solution such as heparin when not in use. When correctly positioned, the tip of the cannula will be position in the vena cava. For longer-term infusions, the femoral vein catheter may be preferable as patency is easier to maintain and the risk of damage to the heart from the catheter is avoided.

Several commercial vendors offer surgical support services and for an additional fee will implant either jugular or femoral catheters. These vendors will typically have a specific methodology for implant, but will accept requests for modifications such as catheter type, exteriorization site, etc. The typical catheter implanted may be manufactured from polyethylene, polypropylene, or silastic. In recent years, manufacturers have developed catheters impregnated or ionically bound with heparin. These materials may help to prolong the life of the catheter.

The useful lifetime for jugular and femoral catheters is quite variable; the lumen of the cannula may eventually become obstructed by a blood clot or fibrous mass. The position of the tip of the catheter is important. Clot formation is less likely to occur if the tip of the catheter is placed in the venous stream rather than in the jugular vein (Popovic and Popovic, 1960). It is recommended for repeated short-term infusions, and when test article is not being infused, a slow infusion of saline will help to prolong the life of the catheter.

Prior to use, the patency of the catheter should be checked by removing the anticoagulant lock, check for blood draw back, and then flushing with saline or Lactated Ringers solution. Alternatively, patency can be checked by injecting 3–6 mg of pentobarbital solution (0.05–0.10 mL of a 60 mg/mL solution) into the catheter (Weeks, 1972). If the catheter is patent, the rat will lose its righting reflex and become ataxic within 10–15 s of injection. The rat will recover in 10–15 min.

Rats will destroy the catheter if it is left unprotected or in easy reach of the forepaws. By exteriorizing the catheter between the scapula, the rat will not be able to chew on the catheter. For the purpose of continuous infusion, several manufacturers have developed tether systems and catheter sheaths made of metal that prevent the animal from chewing on the catheter. These systems typically consist of a jacket with an attached tether through which the catheter is passed. The catheter then attaches to a swivel that prevents the catheter from becoming kinked. The swivel then attaches to a second catheter that can be attached to a syringe or

pump for administration of the test compound (Guo and Zhou, 2003). When performing long-term infusion studies, the effects of the catheter and harness should be considered. Infections, septicemia, a variety of visceral lesions, endothelial lesions, and increased platelet consumption have been observed in cannulated animals (Hysell and Abrams, 1967; Meuleman et al., 1980; Vilageliu et al., 1981). Decreased or erratic weight gains and decreased liver and thymus weights have been observed in tethered animals. These changes may be attributed to the stress involved in chronic tethering of the animals.

An alternative to an exteriorized catheter is to attach a sc port to the catheter that can be accessed via a transcutaneous needle stick. This type of setup helps to prevent infections that can occur with transcutaneous catheters. One of the pitfalls of this sc port is that the port may only be accessed a finite number of time. In addition, care has to be taken to ensure the port and catheter are properly flushed of all test compound and blood as clots can easily form. Administration of small volumes of test compound may be accomplished using a sc implanted osmotic pump. This type of pump is connected to the catheter after being filled with the test compound and implanted in a sc pocket. This allows for continuous administration of small amounts of compound without the need for a jacket and tether system.

#### *Saphenous, Lateral Marginal, and Metatarsal Veins*

These veins in the leg and foot are easily visualized and can be injected without anesthesia; however, assistance is required. Shaving the area over the saphenous or lateral marginal vein makes visualization easier. During injection it is necessary for one technician to restrain the animal and occlude the vessel to cause it to dilate. Wiping the skin over the vein with 70% alcohol or with gauze soaked in hot water will help to dilate the vessel and increase the possibility of success. The second technician then performs the injection, in which a 26–27-gauge needle should be used.

#### *Dorsal Penis Vein*

When administering test article via the dorsal penis vein, it is preferable to use anesthesia. Lightly anesthetizing the animals with an inhaled anesthetic such as isoflurane or $CO_2/O_2$ will prevent the animal from struggling and increase the possibility of a successful injection. This procedure requires two technicians to perform the injection. One technician holds the animal by the skin on the back and the feet and tail. The vertebral column is then hyperextended. The second technician then grasps the tip of the penis between the thumb and forefinger, and injects the test solution into the dorsal vein using a 26–30-gauge needle.

#### *Sublingual Vein*

Although the method of sublingual vein injection has the disadvantage of requiring anesthesia, it only requires one technician. Ideally, the animal should be anesthetized with an inhaled anesthetic such as isoflurane or $CO_2/O_2$, but injectable anesthesia may also be used. The animal should be placed in dorsal recumbency with the head toward the operator. The test compound may be administered by holding the tongue between the thumb and forefinger; using a 26–30-gauge needle, the vein is entered at a very shallow angle and the injection is performed. After completion of the injection, the bleeding can be stopped using direct pressure. Once the bleeding has stopped, a small cotton-wool pledget should be placed over the vein and the tongue placed back in the mouth. The animal will spit the cotton out on regaining consciousness.

### Intraperitoneal Route

Test compounds injected into the peritoneal cavity will be absorbed into the portal circulation and transported to the liver. As a result, the compound will be subjected to the metabolic activity of the liver prior to being circulated to the remainder of the animal. Based on the level of blood flow and circulatory surface area in the peritoneal cavity, compounds injected intraperitoneally (ip) will be absorbed quickly.

Intraperitoneal administration of test compounds in the rat can be performed by one person. The animal should be picked up by the scruff of the neck and back and held firmly in dorsal recumbency. This position will allow for proper access to the peritoneal cavity. The belly of the animal should be visually divided into quadrants and a needle (<21 gauge) should be inserted anteriorly into one of the lower quadrants just lateral to the midline. Aspiration of the syringe prior to injection will help the investigator to determine if the needle is positioned appropriately.

### Intramuscular Route

Intramuscular (im) injection of compounds will result in the rapid absorption into general circulation due to the abundant supply of blood vessels. However, the speed of absorption will not be as fast as with an ip injection. Acceptable sites in the rat are the quadriceps, the thigh, and the triceps. This procedure can be done with one or two technicians. The selected muscle mass should be stabilized with the thumb and forefinger of one hand while restraining the animal and guiding the needle into the muscle with the other hand. A 21-gauge or smaller needle should be used. The needle should be lightly aspirated to ensure the tip of the needle

is not in a blood vessel and then the compound is slowly injected. A slow injection with a minimal volume will help to minimize pain. Approximately 1 mL/kg of solution can be injected per site. If larger volumes are required, multiple injection sites should be used.

## Subcutaneous Route

Absorption following sc injection is typically slower than following im injection. This may be advantageous if a relatively sustained period of absorption is desired. Another advantage of the sc route versus the im route is that a much large volume of test compound can be administered. Five to ten milliliter can be easily injected with little to no discomfort to the animals. This can be beneficial for test compounds that have limited solubility. Suitable sites for injection are the ventral body, the flank, and shoulders. To perform the injection, the skin is grasped between the thumb and forefinger and raised to make a tent. The needle (<20 gauge) is inserted through the skin to make the injection. Injection sites can be varied for multiple-dose studies where the solution is a potential irritant.

To minimize the stress of manipulation and to provide a means for continuous infusion, a perforated cannula or catheter can be implanted transcutaneously. The cannula can then be secured using a tether system similar to that used for iv infusions. Using this system, the test compound can be infused continuously. Mucha (1980) injected sodium pentobarbital directly into the cannula and showed it was absorbed much more rapidly than following injection. An alternate method of infusion of small volumes over an extended period of time is through the use of an osmotic minipump. The pump is filled with the test compound and implanted sc under anesthesia. These pumps are commercially available and offer the advantage of a continuous infusion at a constant rate without the animals being encumbered with the infusion apparatus. This method would work only when the solutions are stable at body temperature for the duration of the infusion period. In recent years several types of absorbable microspheres have been developed that can act as carriers for the test compound.

## Topical Route

The rat has not traditionally been used as a model in skin irritation or sensitization studies. However, the rat has been used in systemic toxicity studies where the skin is used as a portal of entry for whole body exposure or in skin painting studies where the carcinogenic potential is being assessed. In a comparison of absolute absorption rates of several compounds, Bartek et al. (1972) found dermal absorption rates in the rat tended to be slightly lower than in the rabbit, and higher than in the monkey, swine, and human.

Exposure in dermal studies is usually to the anterior dorsal portion of the back. The skin should be shaved weekly or 24 h prior to skin painting. Care should be taken to ensure that the skin is not damaged during shaving, as this can increase the rate of absorption of the test compound. The test area for application should be clearly marked; for repeat dose studies, the area of this site is often 10% of the body area. Usually 0.25–1.0 mL of the test solution is applied in skin painting studies. The amount of a cream or ointment applied will vary with the test compound and desired total dose administered. Dosing is typically performed every day.

The actual dose in a dermal toxicity study is determined by the amount of compound absorbed, therefore factors that influence absorption should be considered. Several design features in a topical study may affect absorption; abraded skin will tend to absorb faster than intact skin; test compound may adhere to or build up at the site of exposure and may impede absorption (and test compound may be chemically changed owing to exposure to air or light); the test compound may be licked or scratched from the site; and the test compound may be ingested by the animal.

Several techniques have been developed to avoid removal or ingestion of the test compound. For acute studies, Rice and Ketterer (1977) described a cable-type restrainer attached to a stainless steel plate. Loops just behind the front and just in front of the hind legs hold the animal immobile. Other methods may be used to reduce stress to the animals and allow the animal mobility. One method is the use of an "Elizabethan" collar. This is a 4–5-cm wide strip of plastic or metal that fits around the neck of the animal. This prevents the animal from being able to turn its head to gain access to its back. Consideration should be given to the use of this method as the collar also may prevent the animal from being able to properly eat from a feed jar. A second method is to wrap the animal with gauze and then with plastic wrap. Care should be taken when wrapping the animal to ensure that the wrap is not too tight. This type of covered exposure may affect the absorption of the test compound. Other types of harness, collars, and acrylic chambers may be used, and the appropriate technique should be chosen based on the intended length of exposure and the efficacy with which the technique can be performed by the technicians.

## Rectal Route

The rectal route is not a routinely used method of administration in toxicology. However, administration

by this route is sometimes required to support drugs given rectally by suppository. For dosing, the animal is held by the base of the tail and a stainless steel, and ball-tipped gavage needle (5 cm) or vinyl tube (6 cm) attached to a syringe is inserted into the rectum. Care must be taken not to damage the rectum when inserting the needle. The syringe should be held lightly: the weight of the needle and syringe propel the needle. The animal can either be awake or anesthetized. If animals are awake, excretion of the unabsorbed test compound may occur. Methods to control excretion have included ligation of the rectum (Nishihata et al., 1984) or various types of septums that are tied or glued in place (DeBoer et al., 1980; Iwamoto and Watanabe, 1985). Anesthetized animals can be placed on an inclined board to retard expulsion.

An important factor in rectally dosing the rat is that the depth of deposition of the test compound will affect the rate of absorption and should be standardized. Drugs subject to extensive first-pass metabolism, such as propranolol and lidocaine, have been found to be much more bioavailable when injected close to the anus rather than in the upper areas of the rectum (DeBoer et al., 1980; Iwamoto and Watanabe, 1985). The reason for this difference in bioavailability appears to be that the venous return in the upper rectal area is through the upper rectal vein that feeds back into the portal circulation and then into the liver. The venous return in the lower rectum is through the lower hemorrhoidal veins and is not connected to the portal system, but goes directly to the inferior vena cava (Iwamoto and Watanabe, 1985).

### Intranasal Route

With the increasing number of drugs being delivered nasally, methods have been developed to support this route. For administration in unanesthetized animals, the appropriate volume of test material is drawn into the tip of a pipette or other appropriate dosing implement. The tip of the dosing implement is placed directly over but not into the nostril to be dosed, and the test material is instilled into the nostril. The animal will aspirate the test material into the nasal passage. This can be repeated for the opposite nostril, or the opposite nostril can be used as a control treatment. In the event that the animal sneezes or the test compound is otherwise expelled, the nostril should be retreated.

### Inhalation Route

Owing to the complexities and equipment involved in generating, maintaining, and measuring appropriate atmospheres, the inhalation study is one of the most technically difficult to perform. This section is not written to provide a complete discussion of the skills necessary to perform an inhalation study, but will deal with the general considerations about the three major steps in exposing rats by the inhalation route: generation of the test atmosphere, exposure of the test animals, and measurement and characterization of the test atmosphere.

### Intratracheal Administration

An alternative to inhalation administration is to instill the test compound into the trachea of the animal. Techniques have been developed to instill the test compound into the trachea safely and repeatedly. To perform the procedure, the animal must first be anesthetized, preferably with a gas anesthesia such as isoflurane. Once anesthetized, a speculum is inserted into the mouth and passed into the trachea. A syringe and needle with a 5-cm piece of tubing is used to instill the test compound. The tubing is then passed over the speculum and the test compound is administered. Volumes should be limited to 2 mL or less of test compound.

## REGIMEN

There are a number of factors that can be manipulated to maximize both administered and absorbed levels of a drug, as well as increasing both tolerance and target tissue specificity. Most of these are beyond the scope of this volume (Gad and Spainhour, 2017), but regimens (how often and at what intervals a test article is administered) are fundamental tools of the experimental animal researcher. Once, twice (bid), or three (tid) times a day are readily available approaches. Likewise, for parenteral routes, an administration can be done quickly (bolus) or over a period of time (infusion).

## END POINT MEASUREMENT TECHNIQUES

### Observations and Physical Examinations

Rats are routinely monitored during toxicology studies as an assessment of their general health and to define the effects of the test article. In acute and subchronic studies, animals may be observed frequently in an effort to define short-term pharmacologic changes induced by the test compound, which may become apparent at peak blood levels. Specifically, in acute toxicology studies clinical observations will help to establish a MTD. In chronic studies, these observations are critical in tracking tumor development and for determining

animals in extremis, which should be euthanized for humane reasons and to prevent autolysis and tissue loss. Arnold et al. (1977) provide a useful description of a clinical assessment program for chronic studies.

Daily observations are performed first thing in the morning and last thing before leaving in the afternoon to assess the health of the animals and identify animals that may be in extremis. In this observation, behavioral status, respiratory signs, skin, eyes, and excretory products are noted. Care should be taken to disturb the animal as little as possible, as this may induce stress and affect the animal's behavior. The animal should be picked up and examined more closely if abnormalities are detected. Special attention should be paid to the amount of feces present, because a decrease in fecal output may be the first signs of a watering system malfunction. In acute studies or where pharmacological effects are expected, animals may be examined continuously or at peak plasma levels.

A more thorough physical examination should be done weekly. Each animal is taken from its cage and placed on an examination table where respiration, behavior, general appearance, and locomotion are observed. The technician should then pick up the animal, examine its body orifices, skin, and coat, and perform a palpation of the trunk and limbs to check for tumors. The detection and tracking of the size and fate of masses (potential tumors) is essential for carcinogenicity studies.

Animals that have experienced severe weight loss over the previous week, a progressive decline in weight over several weeks, or other severe clinical signs should be observed more frequently and marked for possible euthanasia. Body and feeder weights can be measured as part of the physical examination or as a separate function. Performance of these operations as a part of physical examinations will minimize animal handling and potential stress. If the operations are combined, it is important that the physical examinations be done completely and not rushed.

## Neurobehavioral Examination

Neurobehavioral examinations are included in toxicology studies to assess the behavioral and neurological effects of test compounds. These examinations, which may be done as part of acute or repeat dose toxicity studies or studies specifically designed to assess only neurobehavioral effects, typically involve screens consisting of an abbreviated functional observational battery and some measure of locomotor activity (Annau, 1986). The EPA has written guidelines on the design and conduct of these studies (OECD, 1997, 2008). To meet EPA guidelines, the screen is performed prior to the start of treatment, then periodically during the course of treatment. It may be performed as a separate study, on satellite groups of animals in conjunction with the main study, or on animals in the main portion of the toxicology study. The ICH in 2002 adapted guidelines requiring neurobehavioral assessment of all new pharmaceuticals prior to initiation of Phase I clinical trials. To meet ICH guidelines, a more formal functional observational battery should be conducted and may be conducted on main study animals or may be conducted as a separate study. Where initial screens indicate the possibility of a test compound—related change, a more specialized series of tests may be performed to assess the nature of the effect and the extent of the central nervous system involvement. These secondary tests evaluate motor and sensory function as well as cognitive ability. Examples of secondary tests include sensory-evoked potential experiments and schedule-controlled behavior studies. Descriptions of these secondary tests can be found in Annau (1986).

## Functional Observational Battery

The typical observational battery (FOB) includes observation of home-cage and open-field activity as well as measurements of reflexive, physiological, and neuromuscular function (Gad, 1982; Moser et al., 1988; Moser and Ross, 1996) as outlined in Table 2.15. Observations and measurements that have become standard for an FOB evaluation are in Table 2.16. The order of measurement should be consistent, progressing from the least interactive to the most interactive measurements. Home-cage observations are made first. Assessments of posture, clonic movements, tonic movements, and palpebral closure (if the animal's eyes can be seen) are taken prior to removing the animal from its cage. However, it may be necessary to pull the cage from the cage bank or remove the cage cover to see the animal's eyes. The animals are then transferred to the open field. During the transfer, certain physical observations are made. The technician removes the animal from the cage. The technician holds the animal and notes increased or decreased body tone as well as such observations as bite marks, soiled fur appearance, missing toe nails, emaciation (shallow stomach, prominent spinal vertebrae), or death. In addition, observations of lacrimation, palpebral closure, piloerection, exophthalmus, and salivation are also made. The animal is then placed in the open-field apparatus for a set period of time. Measurements of rearing, urination, and defecation are made immediately at the end of the assessment period. Assessment of clonic movements, tonic movements, gait, mobility score, arousal, respiration, stereotypic behavior, and bizarre behavior may be made

**TABLE 2.15** Functional Observational Battery in Rats

| | Number of Animals | |
|---|---|---|
| | **Males** | **Females** |
| Control | 10 | 10 |
| Low dose | 10 | 10 |
| Mid dose | 10 | 10 |
| High dose | 10 | 10 |

**Dosing**: The test article will be administered by the required route.
***Functional Observational Battery***: *FOB evaluations will be conducted prior to dosing, at the estimated time of peak effect, and 24 h postdose. The evaluations are as follows:*
***Home Cage Observations***:
*Assessments of posture, clonic movements, tonic movements, and palpebral closure (if the animal's eyes can be seen) are taken prior to removing the animal from its cage.*
***Handling Observations***:
*Observations of ease of removal, handling reactivity, lacrimation, palpebral closure, piloerection, and salivation are made on removal of the animal from the home cage.*
***Open Field Observations***:
*The animal is placed in the center of an open-field testing box (measuring 20″ × 20″ × 8″). Clean absorbent paper may be used to cover the bottom of the box if required by protocol. Using a stopwatch, the animal's stay in the box is timed for 3 min. Measurements of rearing, urination, and defecation are made immediately at the end of the 3 min. Assessment of clonic movements, tonic movements, gait, mobility score, arousal, vocalization, respiration, stereotypic behavior, and bizarre behavior may be made immediately after the 3 min have ended or the technician may continue to observe the animal for a longer period of time to allow for more accurate assessment.*
***Sensorimotor Observations***:
*The approach response, touch response, click response, and tail-pinch response (stimulus reactivity tests) are performed while the animal is in the open-field apparatus, after the 3 min time period is over and all other measurements have been recorded. The animal is removed from the open field apparatus for the pupil response, righting reflex, thermal response, hind limb splay, and grip strength measurements.*
***Physiological Evaluations***:
*The animal's body weight and rectal temperature is measured and recorded.*
***Clinical Examination***: *Following each FOB assessment (additional observations will be conducted prior to dosing for locomotor activity, if requested).*

immediately after the time period has ended or the technician may continue to observe the animal for a longer period to allow for more accurate assessment. When the open-field assessment has been completed, the animal is removed from the open-field apparatus for the approach response, touch response, click response, tail pinch response, pupil response, righting reflex, thermal response, hind limb splay, and grip strength measurements. After completion of the manipulative assessments, the physiological evaluations are completed.

Procedures used during the performance of an FOB must be standardized because some observations made have a subjective component. If at all possible, a single observer should be used throughout a single study. If not possible, a single observer should conduct all assessments of an animal. In addition, technicians should be blinded to the treatment conditions for each animal.

## Locomotor Activity

Methods used for recording motor activity include direct observation and automated techniques such as photocell devices and mechanical measurements (MacPhail et al., 1989). In direct observations, the observer can make quantitative measurements of the frequency, duration, or sequencing of various motor components of behavior or qualitative records on the presence or absence of certain components of activity. Photocell devices record the number of times an animal interrupts a beam in specially designed chambers. In mechanical chambers, the animal's movements result in a vertical or horizontal displacement of the chamber; records are kept of the chamber's movements. There are advantages and disadvantages of each technique. In direct observation, record can be made of behavior, such as convulsions, which may not be observed when using the photocell or mechanical methods. A disadvantage of the direct observation method is that the animal may be influenced by the presence of the observer. Advantages to the photocell and mechanical methods are that the data are captured electronically, the observer does not have to be present, and the computer system can graphically present the data in the form of lines crossed or a map of the activity.

To make activity determinations, an animal or group of animals is put into an observation or recording chamber and activity is recorded for a specific period of time. Because activity will normally decline over the course of the session, the length of the observation period is important. The EPA guideline specifies that activity should approach asymptotic levels by the last 20% of the session. Haggerty (1989) used a 15-min recording session, accumulating data over three 5-min intervals. Because a large number of environmental conditions can affect motor activity, e.g., sound level, cage design, lighting, temperature, and humidity, or odors, it is important to minimize variations in the test environment.

## Cardiovascular Parameters

Examinations of the cardiovascular system may be scheduled into toxicology studies or performed when the cardiovascular system is a suspected target of the test compound. The ICH has adapted guidelines requiring cardiovascular safety assessment of all new pharmaceuticals prior to initiation of Phase I clinical trials. This guideline recommends that this assessment be conducted in a nonrodent species, but cardiovascular assessment can be performed for screening purposes or as additional support data.

**TABLE 2.16** Functional Observation Battery

| Home Cage | Open Field | Manipulative | Physiological and Neuromuscular |
|---|---|---|---|
| Posture | Rearing | Ease of Removal From Cage | Body Weight |
| Clonic Movements | Urination | Handling Reactivity | Body Temperature |
| Tonic Movements | Defecation | Lacrimation | Hind limb extensor strength |
| Palpebral Closure | Clonic Movements | Palpebral Closure | Grip Strength |
| | Tonic Movements | Piloerection | Hind limb Splay |
| | Gait | Exophthalmus | |
| | Mobility | Salivation | |
| | Ataxia | Approach Response | |
| | Arousal | Touch Response | |
| | Vocalizations | Click Response | |
| | Respiration | Tail Pinch Response | |
| | Stereotypical | Pupil Response | |
| | Bizarre Behavior | Eye blink response | |
| | | Forelimb extension | |
| | | Hind limb extension | |
| | | Righting reflex | |
| | | Thermal Response | |

*From Gad, S.C., 1982. A neuromuscular screen for use in industrial toxicology. J. Toxicol. Environ. Health 9, 691–704; Moser, V.C., McCormick, J.P., Creason, J.P., et al., 1988. Comparison of chlordimeform and carbaryl using a functional observational battery. Fundam. Appl. Toxicol. 11, 189–206; Haggerty, G.C., 1989. Development of tier I neurobehavioral testing capabilities for incorporation into pivotal rodent safety assessment studies. J. Am. Coll. Toxicol. 8, 53–69.*

## Electrocardiography

Although the dog has traditionally been the species of choice in toxicology studies of effects of ECG (also called EKG), research with the rat has progressed and increased over the years. Detweiler (1981) and Detweiler et al. (1981) provide an excellent review of the use of electrocardiography in toxicology studies in the rat. This section will present a general discussion on the aspects of the recording methods and interpretation of the ECG in the rat.

## Recording Methods

### *Restraint*

One of the disadvantages of traditional methods of studying ECGs in rats is that it is difficult to keep the animals still while recording. It is important that the animal remain in a constant position during the procedure using skin leads to avoid muscular artifact in the ECG. Various forms of restraint have been tested, each of which requires acclimation to the procedure prior to evaluation. These methods included restraining the rat in a supine position using rubber gloves (Hundley et al., 1945), pinning the animal to a board, boards with clamps, and plastic tubes with slits on either side, which allow for placement of the electrodes (Zbinden et al., 1980; Spear, 1982). Various forms of anesthesia have also been evaluated.

No conclusion has been reached about the best method of restraint. The basic concerns are that manual methods and physical restraint require acclimation to allow the animal to become accustomed to the procedure, and tracings can be reasonably free of muscular artifact. Also, varying pressures of clamps or handling during the restraint may affect the results. The use of anesthesia has been shown to produce changes in the ECG and the possibility of drug interactions between the anesthetic and the test compound may occur.

### *Position*

The most common positions are the prone or ventral recumbency position when animals are awake and the supine or dorsal recumbency position when anesthetized. Beinfield and Lehr (1956) compared the positions and concluded that the prone position produced an increased R wave and it avoided unfavorable cardiac rotation and an undesirable variation in the projection of the special QRS loop.

### *Tethered*

Robineau (1988) developed an electrode system that can be implanted sc a few days prior to recording. The device has a disconnect that is exteriorized between the scapula. The advantage of this system is that a cable can be connected to the plug and the ECG can be taken in unrestrained rats. The disadvantage of this method is that it should only be used for short-term studies as the implant provides a source for infection in the animal.

### *Leads*

Most investigators use Einthoven's bipolar limb lead system, lead I (right and left foreleg), lead II (right foreleg and left hind leg), and lead III (left foreleg and left hind leg), with and without the augmented unipolar limb leads aVR (right foreleg), aVL (left foreleg), and aVF (one of the hind legs). Because foreleg position can alter the scalar ECG wave amplitudes, investigators must standardize foreleg positions during recording. When implanting telemetry leads, the leads are placed on the right clavical and the most caudal rib on the left side in a modified lead II configuration.

In the past, various types of leads have been used to connect the ECG wires. These included the use of hypodermic needles inserted under the skin, small gauge insulated copper wires wrapped around the shaved distal portion of the limbs, or alligator clips. In addition, platinum-tipped pin electrodes are commercially available, which provide a good quality signal with limited discomfort to the animal.

### *Telemetry*

For the last 10 years, techniques have been developed for monitoring cardiovascular parameters via telemetry in the rat (Kuwahara et al., 1994; Kramer et al., 1995; Ichimaru and Kuwaki, 1998); they have been developed, improved, and used increasingly. Totally implantable battery-operated systems that can monitor several physiological parameters including ECG have been developed. The implants are available with ECG leads that can be positioned in the Lead II configuration for monitoring ECG continuously for extended periods of time in a freely moving animal. As the leads are attached sc to the musculature, the signal is of a higher quality than skin leads. Also, the animal is not affected by the observer, as the animal can be monitored remotely. One of the disadvantages is that these implants are only designed to monitor a single lead.

### *ECG Waveform*

The major points to notice about the rat ECG are that the conventional waves of the mammalian ECG (P, QRS, and T) are all identifiable in the rat ECG, there is no isoelectric line during the electrocardiographic complex, and there is no ST segment. The duration of the standard intervals evaluated in the ECG of the rat are as follows: P, 10−20; PR Interval 35−50; QRS complex 12−25, and QT interval 38−80 ms (Detweiler, 1981). The duration of the intervals is related to the heart rate; as the heart rate increases, the intervals shorten and as the heart rate slows, the intervals prolong. These intervals can also be affected by administration of test compounds and are the basis for the requirement to assess the effects of a test compound on cardiac function. Specifically, prolongation of the QT interval has been correlated with a phenomenon call Torsade de Pointe or sudden cardiac death. Several classes of compounds, such as antihistamine and $Ca^{2+}$ channel blockers have been shown to prolong the QT interval (Gras and Llenas, 1999). Each wave of the ECG represents either a depolarization or repolarization of the atria and ventricles of the heart. For example, the P wave is an electrical representation of the depolarization of the right atria and the T wave is an electrical representation of the repolarization of the left ventricle. Spear (1982) provides a more in-depth discussion of the waveforms and the electrophysiology of the heart. Several computerized systems that are capable of recognizing the independent waveforms and measuring the intervals have been developed. Caution should be taken when using these systems as they are typically programmed to recognize a normal ECG, and the presence of arrhythmias may be missed or interval may not be measured correctly.

### *Heart Rate*

The heart rate can be calculated from standard limb lead ECGs by measuring the distance between the two peaks of the R wave. This distance is then divided by the chart speed (i.e., 50 mm/s) to calculate the RR Interval in seconds. This is then divided into 60 s to calculate the heart rate in beats per minute. Using the formulas below, the heart rate of an RR Interval of 10 mm is measured on a chart printed at 50 mm/s = 300 beats/min.

$$\text{RR Interval (mm/beat)}/\text{Chart Speed (mm/s)} = \text{RR interval (s/beat)}$$

$$(60\ \text{s/min})/\text{RR interval (s/beat)} = \text{Heart Rate (Beats/min)}$$

Many environmental factors can affect the heart rate of the rat, such as excessive manipulation, technicians the animal is not familiar with, new environments, etc. Therefore, when evaluating ECG using a restrained method, it is important to acclimate the animal to the test procedures prior to starting. Detweiler (1981), in a review of the literature, found that published heart rates for rats varied between 250 and 750 beats/min. Awake, restrained adult rats had heart rates from 330 to

600 beats/min and well-acclimated restrained adult animals had heart rates from 250 to 350 beats/min. Heart rate in unrestrained telemetrized animals has been reported to be between 225 and 350 beats/min (Guiol et al., 1992; Zbinden, 1981).

## Blood Pressure

During the conduct of a toxicology study it may be necessary to monitor blood pressure. This can be done using either indirect or direct methods. Caution should be taken when using indirect methods as the values obtained may be variable. The direct method involves the implantation of an arterial cannula for measurement of blood pressure. This method is more reliable, but it has a limited period during which the cannula may remain patent. It is recommended that for definitive assessment of the hemodynamic effects of a test compound, a nonrodent species such as a dog or a nonhuman primate be used.

### *Indirect Measurement*

Indirect methods of blood pressure measurement detect systolic blood pressures by the occlusion of arterial inflow of blood and the subsequent detection of the pressure at which the first arterial pulsation occurs. The two places where indirect measurements can be made on the rat are the tail and the hindpaw.

#### Tail Cuff Method

The tail cuff method monitors pressures in the ventral caudal artery. In this method, the animal is put into a restrainer that allows for free access to the tail. An inflatable cuff is then placed around the base of the tail and the pressure is increased until flow stops. The pressure is then slowly released until flow resumes. The cuff pressure at the time when flow resumes is the systolic blood pressure. Various methods have been used to determine when this occurs.

Because the caudal pulse is rather weak, preheating of the animals in boxes at temperatures of 30–42°C for periods up to 10 min may be necessary to dilate the caudal artery. This technique should be used with caution, as previously discussed changes in body temperature can have widespread effects on the animal and may produce unexpected test compound effects.

The placement and width of the tail cuff is important. There is a gradient in pressure along the caudal artery, which amounts to 4.5 mmHg/cm. For this reason, the cuff should be placed close to the base of the tail and this should be standardized. If multiple reading will be done over time, marking the placement of the cuff with an indelible marker will help to standardize placement. In addition, variation in the width of the rubber tubing can be a source of error. Bunag (1973) found that the most accurate readings were given by a 15-mm cuff; shorter cuffs gave falsely elevated readings and longer cuffs gave low readings.

#### Hindpaw Method

Measurement of blood pressure in the hindpaw does not measure the pressure in a specific vessel. In this method, the animal is placed in a restrainer, as in the tail cuff method. A pressure cuff is placed around the ankle to occlude blood flow and blood pressure is measured as the cuff pressure is released and blood flow returns. As in the tail cuff method, several techniques have been used to determine the return of blood flow. These include visual observation (Griffith, 1934), photoelectric cell (Kersten et al., 1947), and oximeter (Korol and McShane, 1963). The advantage of the photoelectric and oximeter methods is that they do not require preheating to dilate the vessels of the hindpaw. The oximeter method measures mean arterial pressure rather than systolic pressure. None of the indirect measurements are able to evaluate the complete hemodynamic cycle. They evaluate only systolic or mean arterial pressure, but not systolic, diastolic and mean arterial pressure together.

## Direct Measurement

The direct measurement techniques involve the cannulation of an artery with the blood pressure being determined with a manometer or transducer connected to the free end of the cannula. This is true for types of direct measurement including telemetry. Surgery and cannula placement utilize similar techniques as those previously described for placement of a venous catheter. In short, the artery is isolated and a small incision is made in the femoral artery. The catheter is then inserted into the hole and passed into the abdominal aorta. The carotid artery may also be used for this procedure, but care should be taken not to insert too far as the catheter may be passed into the left ventricle of the heart. If this occurs, the blood pressure waveform will change in appearance. The left ventricular waveform has a similar systolic pressure as an arterial blood pressure waveform. But the diastolic pressure is much different. If the catheter has been placed in the left ventricle, the diastolic pressure will be 0 mmHg or slightly negative. If this occurs the catheter should be backed out into the aortic arch.

Where chronic use is desired, cannulas are typically run sc and exteriorized between the scapula or at the back of the head. A carotid artery catheter can be expected to remain patent for 3–5 weeks (Ross, 1977; Andrews et al., 1978), whereas an abdominal aorta

catheter may remain patent for several months. Care should be taken when using arterial catheters for long periods as fibrin deposits can build up on the catheter or clots may form in the catheters. The risk exists that these deposits or clots could be expelled during the flushing of the catheter or during normal movement of the animal. These clots or deposit once free may occlude other vessels downstream and in the case of a carotid catheter may cause a stroke to occur.

## Blood Collection Techniques

Blood samples are routinely collected in safety studies to determine: (1) direct test compound effects on the blood or bone marrow, (2) effects on other organs as indicated by the contents of the blood, for instance, leakage enzymes such as aspartate aminotransferase (AST), and (3) blood levels of the test compound or its metabolites. A variety of techniques have been described for the collection of blood from the rat. The choice of a specific technique may depend on factors such as: (1) the volume to be collected, (2) if the animal is to survive the procedure, (3) the frequency with which samples will need to be collected, (4) whether anesthetics can be used, (5) likelihood of the animal surviving the procedure, and (6) the impact of organ damage resulting from the procedure. An adult rat has a blood volume of about 50 mL/kg; approximately 10% of the total blood volume can be collected from the rat in a single draw without adversely affecting hematology parameters. For longer-term studies, 10 mL/kg per 2 weeks is a reliable guideline for volume of blood drawn. If these volumes are exceeded, hematocrit and red cell mass may be reduced on evaluation (Diehl et al., 2001).

Technique, anesthetic used for blood draws, and treatment of the animals (i.e., fasted or not fasted) should be standardized throughout a study if repeated samples are being taken. The technique, anesthetic, and handling of the animal may produce effects on the hematology or clinical chemistry parameters to be evaluated.

### *Retro-Orbital Plexus*

The retro-orbital plexus is a commonly used site for periodic sampling during the course of a study. This method has been shown to be a reliable method for the repeated collection of blood samples. Collection from this site should always be conducted under anesthesia to reduce pain and stress to the animal. Light anesthesia with a mixture of carbon dioxide and oxygen will minimize struggling of the animal and will help to ensure a quick collection with little injury to the animal.

Blood is collected using a microcapillary tube or the fine end of a Pasteur pipet. The tube is inserted into the orbit of the eye at an anterior angle formed by the lids and the nictitating membrane. A slight thrust past the eyeball will make the tube enter the slightly resistant horny membrane of the sinus. The tube may be rotated slightly as it is inserted. Once the sinus has been punctured, blood will fill the tube. Once the tube is filled, the blood may be allowed to drip out of the end of the tube into an appropriate collection tube. If the flow stops, the tube may be pulled out or advanced slightly to reestablish flow.

In the hands of an experienced technician, there is minimal risk to the animal. Studies have shown that repeated collection of blood from the retro-orbital sinus can produce histological and behavioral changes that may require an animal to be removed from study (McGee and Maronpot, 1979; van Heck, 1998). Several serious side effects of this collection method have been documented. These include retro-orbital hemorrhage, corneal ulceration, keratitis, pannus formation, damage to the optic nerve, and fracture of the orbital bones. When designing the study, the method of blood collection should be taken into consideration. If an important end point of the study is ophthalmological examinations, this method should not be used. In addition, anesthetization of the animals may affect other study end points.

### *Tail*

A tail vein bleed offers a visible target and is of minimal risk to the animal. Blood will flow faster if the tail has been warmed causing vasodilation. This may be accomplished by dipping the tail in warm water (40–45°C), placing the animal in a warming cabinet for 5–10 min, or warming the tail with a heat lamp. This method does not require that the animal to be anesthetized, but the animal should be restrained such that the tail is held immobile. Several methods may be employed to collect blood from the tail. These include clipping the end of the tail off, vein puncture, or artery puncture.

### *Tail Clip*

For this method, the animal may be lightly anesthetized or placed in a restraint tube. To collect blood, 2–3 mm of the distal part of the tail is amputated with sharp scissors or a scalpel blade. Blood of 3–4 mL can be collected in 20–30 s from a 200–250 g rat. When collection has been completed, the cut surface may be cauterized with a hot spatula or glass rod. This method produces a reliable volume of blood, but it should not be used for repeated sampling over extended periods of time.

### *Venipuncture*

Animals should be restrained in a holder that allows complete access to the tail. The tail should then be cleaned and pressure may be applied to the base of the tail causing the vein to dilate. The vein should be punctured using a 21-gauge needle, and the blood can either be slowly withdrawn into a syringe through the needle or the needle may be removed and the tail allowed to bleed freely. The use of a butterfly needle may help facilitate the collection of blood as it is less likely to be dislodged if the tail moves. Collection of blood from the tail vein typically yields samples of between 0.5 and 1.0 mL. On completion of sample collection the needle should be withdrawn and pressure is applied to the tail to stop the bleeding (Frank et al., 1991).

### *Arterial Puncture*

This method is conducted in a similar manner to a venipuncture in that the animal is placed in an appropriate restraint tube and the tail is cleaned. A 21-gauge needle is then inserted into the artery in the midventral surface of the tail close to the distal end. The blood is then withdrawn into a syringe or allowed to flow freely into a collection tube. The animal's blood pressure will ensure whether the blood continues to flow until pressure is applied to the wound.

### *Cardiac Puncture*

This method should always be performed under general anesthesia and should not be used as a survival collection technique. This technique offers a rapid method for collection of a large volume of blood from the rat. It is possible to collect between 5 and 7 mL from a 300–350 g rat using this technique, and it is possible to exsanguinate the animal using this method.

To collect blood using this method the animal should be anesthetized with a combination of carbon dioxide and oxygen or carbon dioxide alone. The animal is then placed in dorsal recumbency and the heart is located. The heart may be located by placing the index finger over the fourth and fifth left ribs and the thumb on the right side of the thorax. The collection needle (25–26 gauge, 1–2-cm long) should be inserted at a 45-degree angle into the heart. Once the needle is introduced, the syringe should be aspirated slightly to produce a vacuum. The needle is then advanced until blood is obtained.

### *Abdominal Aorta and Vena Cava*

Collection from the abdominal aorta offers a convenient way to exsanguinate an animal and obtain a maximal amount of blood (Popovic and Popovic, 1960). To perform the procedure, the animal is anesthetized and the aorta exposed by dissection. A section of the aorta distal to the diaphragm is then exposed and the proximal end is clamped. The aorta is then cut and the distal end placed in a collection tube and the clamp is released. This method will allow for collection of a maximal amount of blood in a short period of time. This method should only be used as a terminal procedure. Alternatively, the aorta may be accessed using a needle or a butterfly needle and samples may then be collected into a syringe.

Winsett et al. (1985) described a method of repeated sampling from the vena cava of conscious rats. An assistant holds that animal while the operator grasps the animal just below the last rib. The needle is inserted 1 cm to the right of the spinous process of the first lumbar vertebra at a 45 degrees angle until the needle touches the bone. The needle is slightly withdrawn and then advanced at a slightly shallower angle to miss the bone and access the vena cava. The maneuver of first identifying the bone is essential to the procedure. This procedure may be used for repeated collection, but care should be taken to ensure the animal does not struggle during the collection. If the animal struggles, the needle may lacerate the vena cava causing the death of the animal.

### *Jugular Vein*

The jugular vein provides a means for chronic blood sampling that is of low risk to the animals' health. This method does not require the use of anesthesia and can be accomplished through the proper restraint and positioning of the animal.

The unanesthetized method requires two technicians to perform the collection. The animal is placed on a restraint board in dorsal recumbency. The forelimbs are tied down to the board and one technician holds the hind limbs of the animal. The second technician grasps the animals head and turns it down and away from the desired collection site, right or left jugular. The needle (21 gauge) is then inserted into the middle of the triangle formed by the neck, shoulder, and clavicle, parallel to the body. As the needle is inserted, the syringe is aspirated until blood is observed in the syringe. The collection is then completed and the site is held off for approximately 30 s. It is important that the technician holding the hind limbs of the animal continually observe the respiratory rate of the animal. If the head is turned too far or is held in the wrong position for too long, the animal may go into respiratory distress. This method may be used for repeated collections with a high level of success.

Alternatively, the animal may be anesthetized and the jugular vein may be surgically exposed. The animal should be prepared using standard aseptic technique and the ventral neck should be shaved. The jugular vein may be exposed by incision of the skin and

dissection of the sc tissues. A needle (20 gauge) may then be inserted into the vein through the pectoral muscle, directing the needle toward the head. Inserting the needle through the muscle will help to stabilize the needle during the collection. Once the needle is in place, the blood should flow freely with little to no aspiration. Care should be taken when aspirating the syringe as too much pressure will cause the vein to collapse. Once the collection is complete, the incision can be closed with a wound clip. This method may be used as an alternative if the animal does not require serial bleeds or as a method to replace a terminal bleed. If the procedure is used for a terminal bleed, strict aseptic technique is not necessary.

#### *Proximal Saphenous and Metatarsal Vein*

A small amount of blood (0.1—0.2 mL) can be collected from an animal at minimal risk to their health utilizing the proximal saphenous and metatarsal veins. No anesthesia is required.

#### *Proximal Saphenous Vein*

The inner aspect of the thigh of the hind limb should be shaved free of hair. While one technician holds the animal and compresses the inguinal area to dilate the vein, a second technician creates a longitudinal nick in the vein with a 20-gauge needle or a hematocrit lancet. The blood can then be collected into heparinized capillary tubes. This method works well for repeated sampling of small amounts of blood but would not be appropriate for blood volumes required for evaluation of clinical pathology parameters.

#### *Metatarsal Vein*

This procedure can be conducted with or without an assistant. The animal is restrained and a nick is made in the vessel with a needle. The blood can then be collected into a capillary tube or through the needle into a syringe.

#### *Sublingual Vein*

When using the sublingual vein for blood sampling, the animal should be anesthetized and then cradled in the palm of the hand. By holding the animal's head between the thumb and index fingers, the head can be stretched back and the skin of the face pulled backward. This will force the mouth open and the tongue against the palate. The right or left vein should be cut with iris scissors and the animal held such that blood drips into the collection tube. The bleeding can then be stopped by applying pressure with a gauze pad or cotton-tipped applicator.

#### *Decapitation*

Decapitation should only be performed by trained technicians with the appropriate equipment. There are several commercially available small animal guillotines that should be used to perform this technique. This technique is appropriate when a maximal blood volume is desired and contamination of the sample is not considered to be an issue. To perform this method, the head is first removed and the animal is held over the collection vessel and arterial and venous blood is allowed to drain from the body.

#### *Cannulation*

Although the blood collection methods described above will provide sufficient volumes and quality of sample for the majority of toxicology studies, specific protocols may require blood to be sampled from animals that have been subjected to a minimum of handling or from specific sites within the body of the animal. Cannulation of a specific artery or vein will typically meet this requirement, though in rodents, the usable life span of the cannula is limited by the length of time the cannula remains patent. Yoburn et al. (1984) compared jugular, carotid, and femoral cannulas for long-term sampling of blood. They found the femoral artery cannula was preferable in terms of patency and postsurgical weight loss. Collection from a cannula is the same regardless of implant site. The cannula is typically exteriorized between the animal's scapula and a stylet is inserted into the end of the cannula. For the purpose of collection, the stylet is removed and a needle is inserted into the cannula with a syringe attached. The heparin lock is then drawn out of the cannula until blood is observed in the syringe. The syringe is then removed from the needle and a new syringe is used to collect the sample. The collection syringe is then removed from the needle and replaced with a syringe filled with the desired solution for locking the cannula, typically a heparin dextrose solution. Once the cannula is flushed and locked with the heparin solution, the stylet is replaced. Using the appropriate technique for flushing and locking the cannula is the key to maintaining the patency of the cannula.

#### *Jugular Vein*

The cannulation procedure is the same as previously described for infusion techniques. The cannula has been found to remain patent for a variable period of time, and the length of time the cannula remains patent is directly related to the skill of the technician collecting the samples. It is important to remember that a cannula placed in the venous system can easily develop clots in the cannula if not flushed and locked properly. Various methods of anchoring the cannula to the rats back or head have been developed for ease of sampling or as a connection point for continuous infusion. Each of these methods has been developed such that the exteriorized

cannula is positioned so that the animal cannot gain access to it.

### *Inferior Vena Cava*

The inferior vena cava appears to provide a site for long-lived cannulas. The cannula can be surgically placed either directly into the vena cava through an abdominal surgery or can be advanced into the vena cava from the femoral vein. Either way, the cannula has been shown to remain patent for months (Kaufman, 1980). In either implantation, the cannula is then tunneled sc and exteriorized between the scapula in the same manner as the jugular vein cannula.

### *Abdominal Aorta*

The most common method for placing a cannula in the abdominal aorta is via the femoral artery. The cannula is inserted into the femoral artery and advanced to the level of the just above the kidneys. The opposite end of the cannula is then tunneled to and exteriorized between the scapula. This is a minor surgical procedure in which the animal recovers easily. Similar methods are employed when collecting blood from the arterial catheter as those used for the venous catheter. Caution should be taken when removing the stylet from the cannula, as a cannula in the arterial system is under pressure. If not properly prepared, the animal could quickly lose a large amount of blood. It is recommended that the technician place a clamp on the cannula prior to removal of the stylet. Once the blood collection is complete, the cannula should be thoroughly flushed with saline prior to locking the cannula. The tip of an arterial cannula is positioned such that it is against the flow of blood. Because of this, clots and fibrin deposits develop easily on the end of the cannula limiting the usable life.

### *Subcutaneous Ports*

An exteriorized cannula is a source of contamination, infection, and is subject to destruction by the animal or other animals if group housed. Several types of sc ports have been developed for implantation along with the cannula. These ports are designed such that test materials may be injected or infused through them or blood may be collected from the port. In most cases, the port is implanted sc on the dorsal side of the animal. As with exteriorized cannula, the skill of the technician accessing, collecting samples, flushing, and locking the port directly affects the usable life of the port.

## Urine Collection

Urine is generally collected in toxicology studies to assess kidney function. The most common method used for urine collection is a commercially available stainless steel cage. The cage is designed such that the urine and feces are separated by a cone-shaped device. The urine drains off the collecting walls into a tube and the feces fall into an inverted cone. Food and water are made available in such a way that the urine will not be contaminated. Although this type of cage produces urine of acceptable quality for normal urinalysis, the sample may be contaminated with hair or feces. Other methods for urine collection in the rat include cystocentesis, which involves a needle stick into the bladder, and cannulation of the bladder.

## Necropsy

The necropsy is the link between antemortem findings and histological observation. It is an essential portion of the toxicology study, and because a necropsy will involve the processing of a large number of animals, it is important that the procedure is well planned (Black, 1986). At a prenecropsy meeting involving the pathologist, prosectors, and the study director, necropsy responsibilities can be discussed. Additionally, the study director can summarize clinical findings and potential target organs. The prosectors should be familiar with the protocol and amendments for the study involving the animals being necropsied. The protocol should clearly state which tissues are to be collected and weighed and how the tissues should be preserved. During the necropsy, devices such as checklists or prelabeled compartmentalized trays should be present to ensure that all required organs are taken and weighed. In recent years commercially available computer software has been developed to assist in the collection and weighing of tissues at necropsy. This is an electronic copy of the checklist, but it also provides a method for recording observations in a consistent manner. Copies of the last clinical observations should be present at the necropsy, so the prosectors are alerted to lesions that may be present and require special attention. Palpation records are particularly important at carcinogenicity study necropsies to ensure that all masses detected at the last examination are confirmed and collected.

The necropsy will involve a check of animal identification and sex, an external examination of the animal, an in situ examination of all tissues and organs (prior to dissection, and the collecting and weighing of the required tissues).

## SUMMARY

In summary, there are several advantages to the use of the rat in toxicology studies. Because of its widespread use in many fields of biology, there is a large historical

database of information about the anatomy and needs of the species. This knowledge, along with information about the species' metabolism and response to toxicants, has shown the rat to be a generally good model for the prediction of the human response to toxicants. Rats have a life span of 24–30 months, which is convenient for chronic toxicity and carcinogenicity studies where animals need to be exposed for the majority of their lifetime. The short gestation time and large litter size make the rat a good model for reproductive studies. The development of specific pathogen-free rats and improvement in husbandry has eliminated most of the disease outbreaks that may have introduced variability into a study. The lack of an emetic response allows for the testing of higher dosages of compound that may cause vomiting in other species. The small size of the rat is useful in that a large number can be housed economically. The size is also useful in that smaller amounts of test compound are required to gain maximal exposure.

The relatively small size of the rat is also one of its major disadvantages. The amount of blood that can be taken from the animal is limited, thus limiting the number of parameters that can be investigated or the number of toxicokinetic samples that can be collected from a single animal. This problem can be overcome by adding additional animals in interim sacrifice groups or by collecting toxicokinetic samples from cohorts of animals at different times. In most cases it is recommended that toxicokinetic samples be collected from satellite animals and not from the main study animals being used for evaluation of toxicity. However, an increased number of animals means increased work in the conduct of the study. The small size and relatively active nature of the rats makes some procedures, such as iv dosing or collection of electrocardiograms, difficult. These issues have been overcome with the use of suitable restrainers or in some cases, anesthesia. The rat has been used successfully in toxicology research for close to a century and will continue to be used for the foreseeable future.

## The Mouse

### *Use in Toxicological Research*

As discussed earlier, the choice of a species for toxicity testing is based on consideration of a range of variables. Ideally, if toxicity testing is intended to provide information on the safety of a test article in or by humans, the species chosen for testing should be most similar to humans in the way it handles the test article pharmacodynamically. Substantial differences in absorption, distribution, metabolism, or elimination between test species and the target species, e.g., humans, will reduce the predictive value of the test results. From a practical standpoint, often the pharmacokinetics is unknown in humans or the variety of available test species at the time of species selection. For this reason, testing is usually conducted in at least two species. Generally, one of those species is usually a rodent and one a nonrodent.

Mice have many advantages as test animals for toxicity testing. They are small, relatively economical to obtain, house, and care for, and they are generally easy to handle. Mice are generally more economical than rats in these respects. Although mice may attempt to escape or bite handlers, with regular, gentle handling they are easily managed. Other advantages of the species include a short gestation period and a short natural life span. These characteristics allow studies that include evaluation of reproductive performance or exposure to a test article for periods approaching the expected life span (e.g., evaluation of carcinogenic potential) to be conducted in a practical time frame. High quality, healthy mice are available from reliable commercial suppliers. Many genetically well-defined highly inbred, specifically or randomly outbred strains are available. Mice have been used in biomedical research for hundreds of years, and because of this, many technical procedures have been developed for use with the species, and a vast body of historical data is available for most strains. This historical database includes information on optimal nutritional and housing requirements in addition to data such as the expected background incidence of various diseases and types of tumors in untreated animals, and is continuously being added to (Blackwell et al., 1995). An additional advantage for mice, particularly when testing highly humanized biological products, is that transgenic mice that have the gene encoding the specific human pharmacodynamic drug receptor can now be readily developed.

There are also disadvantages to using mice, and most are related to the small size of the animal and the limits that this imposes. The smaller size and higher metabolic rate compared with the rat renders the species less hearty than rats. Deviations in environmental conditions such as an air conditioning failure or failure in an automatic watering system typically have more severe effects on the smaller species such as mice than the same deviations have on rats. Owing to their high level of natural activity, most strains of mice will not become as docile or easy to handle as rats that have received equivalent handling. Small size often precludes or renders more difficult a number of procedures that are commonly conducted in toxicity testing, such as the collection of large samples or repeated samples of blood and urine, electrocardiographic evaluation, and some necropsy evaluations. The FDA provides Human Equivalent Dose interspecies conversion factors for converting most animal model NOAELs or such to a human

equivalent. The conversion factor for the mouse is 12.1, meaning an observed NOAEL in a mouse of 12.1 mg/kg/day would be converted to 1.00 mg/kg/day for conservative human administration. This relatively high value imposes a de facto penalty on using mice as one of the species in a safety assessment.

This section will provide brief summaries of some of the normal physiological values and salient features of the species and some of the specific strains that may be useful in selecting an appropriate species and strain for toxicity testing.

### Normal Physiological Values

Selected normal physiological values for mice are shown in Tables 2.17 and 2.18. Median survival of a number of groups of Charles River CD-I outbred mice is shown in Table 2.19.

These normal values will vary depending on the strain of mouse, supplier, condition at arrival, type of feed, environmental and housing conditions, and, in some cases, time of year. These data should be considered as a reference but will not necessarily represent experience in any particular laboratory.

**TABLE 2.17** Normal Physiological Values General and Reproductive

| | |
|---|---|
| ***GENERAL*** | |
| Life span | |
| Average | 1–3 years |
| Maximum reported | 4 years |
| Adult weight | |
| Male | 20–40 g |
| Female | 18–40 g |
| Surface area | 0.03–0.06 $cm^2$ |
| Chromosome number (diploid) | 40 |
| Food consumption | 4–5 g/day |
| Water consumption | 5–8 mL/day ad libitum |
| Body temperature | 36.5°C |
| Oxygen consumption | 1.69 mL/g/h |
| ***REPRODUCTIVE*** | |
| Age, sexual maturity | |
| Male | 50 days (20–35 g) |
| Female | 18–40 g |
| Breeding season | Continuous, cyclic |
| Estrus cycle | 4–5 days |
| Gestation period | |
| Average | 19 days |
| Range | 17–21 days |
| Litter size | |
| Average | 12 |
| Range | 1–23 |
| Birth weight | 1.5 g |
| Age begin dry food | 10 days |
| Age at weaning | 16–21 days (10–12 g) |

*Data derived from Fox, J.G., Bennett, S.W., 2015. Biology and diseases of mice. In: Fox, J.G., Anderson, L., Otto, G., Prichett-Corning, K., Wary, M. (Eds.), Laboratory Animal Medicine. Academic Press, New York, pp. 31–89.*

## Species Differences

Mice are similar to other common laboratory animal species and to humans in many ways, yet the differences should not be underestimated and must be understood. Mice have a high metabolic rate compared to other species. This fact alone may result in increased or decreased toxicity of a test article, depending on the specific mechanism of intoxication. In many cases, high metabolic rate may be associated with rapid absorption, distribution, metabolism, and elimination of a test article. It may also lead to higher systemic $C_{max}$ levels of toxicants such as reactive oxygen species. Mice are obligate nose breathers and have more convoluted nasal passages than humans. This may result in an excess of respirable test article deposited in the nasal passages, resulting in either increased or decreased relative toxicity, depending on the most critical site of absorption. The small size of the mouse compared to other common laboratory species offers a significant advantage if the test article is expensive or in short supply. As an approximation, a mouse weighs about 10% as much as a rat, about 5% as much as a guinea pig, about 1% as much as a rabbit, and less than 1% as much as a dog or primate. Material requirements to administer equivalent dose levels are usually proportional to body weight, so the test article savings associated with the mouse are evident. The small size of a mouse results in high surface area to body mass ratio, which in turn causes the mouse to be relatively intolerant of thermal and water balance stresses. The kidneys of a mouse have about twice the glomerular filtering surface per gram of body weight as a rat, and owing to the specific architecture of the murine kidney, they are capable of producing urine that is about four times as concentrated as the highest attainable human concentrations (Fox and Bennett, 2015). These characteristics of renal architecture and function may be important to the toxicity of some test articles. Mice differ from most species by the formation of a persistent vaginal plug after mating. The presence of a vaginal plug is easily detected, is considered evidence

**TABLE 2.18** Normal Physiological Values Cardiovascular and Respiratory

| | |
|---|---|
| ***CARDIOVASCULAR*** | |
| Heart rate | |
| Average | 600/min |
| Range | 320–800/min |
| Blood pressure | |
| Systolic | 113–160 mmHg |
| Diastolic | 102–110 mmHg |
| Blood volume | |
| Plasma | 45 mL/kg |
| Whole | 78 mL/kg |
| Hematocrit | 41.5% |
| RBC life span | 20–30 days |
| RBC diameter | 6.6 microns |
| Plasma pH | 7.2–7.4 |
| ***RESPIRATORY*** | |
| Rate | |
| Average | 163/min |
| Range | 320–800/min |
| Tidal volume | |
| Average | 0.18 mL |
| Range | 0.09–0.38 mL |
| Minute volume | |
| Average | 24 mL/min |
| Range | 11–36 mL/min |

*RBC*, red blood cell.
*Data derived from Fox, J.G., Bennett, S.W., 2015. Biology and diseases of mice. In: Fox, J.G., Anderson, L., Otto, G., Prichett-Corning, K., Wary, M. (Eds.), Laboratory Animal Medicine. Academic Press, New York, pp. 31–89, and from the Animal Diet Reference Guide, Purina Mills, Inc. (1987).*

**TABLE 2.19** Median Survival of 16 Groups of Control Mice (%)

| | Period of Time on Study (months) | | | |
|---|---|---|---|---|
| **Sex** | **6 (%)** | **12 (%)** | **18 (%)** | **21 (%)** |
| Male | 98 | 91 | 63 | 46 |
| Female | 98 | 95 | 74 | 68 |

Data represent median survival of Charles River CD-I outbred albino mice enrolled in 24-month chronic toxicity studies at pharmaceutical or contract toxicology laboratories.
*Adapted from Lang, P.L., February 1989a. Spontaneous Neoplastic Lesions in the B6C3F,/CrIBR Mouse. Charles River Monograph. Charles River Laboratories, Wilmington, Massachusetts; Lang, P.L., Fall 1989b. Survival of CrI: CD-I BR Mice during Chronic Toxicology Studies. Charles River Laboratories Reference Paper. Wilmington, Massachusetts.*

**TABLE 2.20** Normal Body Weights in Grams of Selected Strains of Mice

| | Outbred Strains | | | | | | Inbred Strains | | | | | | Hybrid | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD-1 | | CF-I | | CFW | | C3H | | C57BU6 | | BALB/c | | B6C3FI | |
| **Age (days)** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| 21 | 12 | 11 | 12 | 11 | 9 | 9 | – | – | – | – | – | – | – | – |
| 28 | 20 | 18 | 18 | 17 | 16 | 13 | 17 | 16 | 14 | 13 | 16 | 14 | 16 | 14 |
| 35 | 27 | 22 | 24 | 21 | 19 | 17 | 18 | 17 | 17 | 14 | 17 | 16 | 20 | 17 |
| 42 | 30 | 24 | 27 | 22 | 24 | 20 | 20 | 18 | 19 | 16 | 18 | 17 | 22 | 18 |
| 49 | 33 | 26 | 28 | 24 | 27 | 22 | 24 | 23 | 21 | 17 | 20 | 18 | 24 | 19 |
| 56 | 35 | 27 | 30 | 26 | 28 | 23 | 27 | 26 | 22 | 18 | 21 | 19 | 26 | 21 |

*Data derived from Charles River Growth Charts.*

of mating, and is a useful characteristic during the conduct of reproductive studies.

It is also frequently the case in pharmaceutical research and development that the nonclinical efficacy model for a new drug is in the mouse, making it the natural choice for rodent evaluation of the drug.

## Strain Differences

In addition to differences between mice and other species, there are important differences among different strains of mice. The appropriate choice of a strain of mice for a particular toxicity study should consider the specific objectives of the study and the specific characteristics of candidate strains that might assist or hinder in achieving those study objectives.

One difference among strains is in the normal body weights of various strains at different ages. These differences are summarized for selected strains available from the Charles River Breeding Laboratories in Table 2.20. Outbred strains tend to be larger at maturity than inbred strains, with the CD-I strain reaching the highest mean weights at 56 days of age of those strains in Table 2.20. The CF1 strain has been reported to be highly resistant to mouse typhoid and to be relatively resistant to salmonellosis (Hill, 1981). Nude or athymic strains of mice are more sensitive to tumor development than heterozygous strains. These sensitive strains develop the same types of tumors as those seen in more conventional strains, but the incidences are higher and the latency periods shorter. There is a wide spectrum of susceptibility to spontaneous lung tumors in various strains of mice, and evidence suggests that there is a high correlation between spontaneous incidence and chemical inducibility in those various strains (Shimkin and Stoner, 1975).

The inbred strain A mouse appears to be the most susceptible to lung tumors and forms the basis of a

**TABLE 2.21** Incidence of Spontaneously Occurring Neoplastic Lesions

| | CD-I | | B6C3Fl | |
|---|---|---|---|---|
| **Location and Lesion** | **M** | **F** | **M** | **F** |
| ***LYMPHORETICULAR TUMORS*** | | | | |
| Lymphosarcoma | | | 6.0 | 12.0 |
| Lymphocytic leukemia | | | 1.3 | 1.4 |
| Lymphoma | 3.7 | 9.9 | | |
| Histiocytic lymphoma | | | 0.5 | 1.4 |
| Histiocytic sarcoma | | | 0.1 | 1.4 |
| Lymphoblastic lymphoma | 1.1 | 1.7 | 0.2 | 0.1 |
| Lymphocytic lymphoma | 2.6 | 1.7 | 0.6 | 1.7 |
| Reticulum cell sarcoma | 2.6 | 5.1 | 0.4 | 0.2 |
| ***SKIN/SUBCUTIS*** | | | | |
| Fibrosarcoma | 0.2 | 0.5 | 1.0 | 0.5 |
| Mammary gland | | | | |
| Adenocarcinoma | | 1.7 | | 0.9 |
| ***LUNG*** | | | | |
| Bronchiolar/alveolar adenoma | 4.0 | 2.9 | 8.3 | 3.3 |
| Bronchiolar/alveolar carcinoma | 3.5 | 3.1 | 1.9 | 0.6 |
| Alveolar type II carcinoma | 11.7 | 13.9 | 0.2 | 0.1 |
| Alveolar type II adenoma | | | 2.5 | 1.2 |
| Adenoma | | | 1.2 | 0.7 |
| ***LIVER*** | | | | |
| Nodular hepatocellular proliferation | 5.4 | 1.7 | 0.5 | 0.1 |
| Hepatocellular adenoma | 5.6 | 0.8 | 17.2 | 7.1 |
| Hepatocellular carcinoma | 7.3 | 1.0 | 13.2 | 2.4 |
| Hemangioma | 1.0 | 1.2 | 0.7 | 0.3 |
| Hemangiosarcoma | 1.0 | 0.2 | 0.5 | 0.1 |
| ***REPRODUCTIVE SYSTEM*** | | | | |
| Ovary | | | | |
| Cystadenoma | | 1.1 | | 0.3 |
| ***UTERUS*** | | | | |
| Endometrial stromal proliferation | | 3.3 | | 2.9 |
| Endometrial sarcoma | | 1.9 | | 0.6 |
| Leiomyoma | | 1.0 | | |
| Leiomyosarcoma | | 1.0 | | 0.3 |
| Hemangioma | | 1.0 | | 0.9 |
| ***PITUITARY*** | | | | |
| Adenoma | | 3.4 | 0.3 | 7.9 |
| ***THYROID GLAND*** | | | | |
| Follicular cell adenoma | 0.2 | | 0.8 | 2.4 |
| ***ADRENAL*** | | | | |
| Cortical adenoma | 8.6 | 1.0 | 0.4 | 0.3 |
| ***HARDERIAN GLAND*** | | | | |
| Cystadenoma | | | 1.9 | 1.6 |
| Adenoma | | | 1.5 | 0.6 |

Lesions occurring at spontaneous incidence of ~:1% in either sex of Charles River CD-1 or 136C3171 mice strain.
*Data from Charles River Breeding Laboratories, compiled from control animals on 24-month studies completed between 1978 and 1986.*

lung tumor bioassay, with tumors inducible within 8 weeks or less of treatment. Susceptibility of various strains to the initiation and/or promotion of skin tumors has also been shown to differ greatly (Chouroulinkov et al., 1988; Steinel and Baker, 1988). The incidences of selected spontaneously occurring neoplastic lesions in CD-I (outbred) and B60171 (hybrid) strains are compared in Table 2.21.

The number of strain-related differences in susceptibility to various test articles and environmental conditions exceeds the scope of this chapter, but additional information is available (Nebert et al., 1982).

## STUDY DESIGNS

Most toxicity and teratology studies conducted in mice are designed to provide information on potential human toxicity. Test substances are typically administered by the expected route of human exposure. A pharmaceutical product that is intended for oral administration (tablet, capsule, solution, or suspension) or a food additive would generally be administered by the oral route. Oral administration to mice is usually accomplished by administration of a solution or suspension by oral gavage, by mixture of the test substance with the diet, or less commonly added to the drinking water.

The specific design of toxicity studies should be tailored to the objective to be achieved and to any specific characteristics of the test substance. Many features of study design will be predicated on guidelines and practices of regulatory agencies such as the FDA or EPA in the United States or their counterparts in other countries to which the results of the study are submitted

in support of a safety claim. Recommendations for study length (duration of dosing) fall in this category.

Toxicity studies are usually conducted in order of increasing duration of dosing, beginning with acute toxicity studies. When this regimen is followed, each study provides progressively more useful information for the selection of doses for the next, longer study.

## Acute Toxicity Studies

Acute toxicity studies are conducted to evaluate the effects of a single substance. Usually each animal receives a single dose of the test substance in this study design. On rare occasion, repeated doses may be administered, but in any event, all doses are administered within 24 h or less. Historically, a primary objective of acute toxicity testing was to determine an $LD_{50}$ dose, or that dose which would be lethal to 50% of the animals treated. To achieve this objective, groups of mice, often numbering 10 or more per sex, are treated with a single dose of the test substance. Depending on the rate of survival in the initial group(s), additional groups are added to the study at higher and/or lower doses such that most animals that receive the highest doses die and most that receive the lowest doses survive. Survival is assessed at some predetermined interval after dosing, usually 7 or 14 days, but occasionally as early as 24 h. The resultant dose–response data can be analyzed by a statistical method such as probit analysis (Finney, 1971) to provide an estimate of the median lethal dose ($LD_{50}$) and some measure of the precision of that estimate, such as the 95% fiducial limits. There are very few scientifically valid reasons to include determination of the $LD_{50}$ as a significant objective of acute toxicity testing, and most regulatory agencies have dropped their requirements for a specific value for the $LD_{50}$, and animal welfare considerations preclude the use of the large numbers of animals previously required.

A more contemporary design for acute toxicity testing attempts to derive a maximum amount of information from a minimum number of animals. Study objectives include determination of the most important clinical signs attributable to high doses of the test substance, time of onset and remission of those signs, possible determination of a minimum lethal dosage, and in the event of lethality, the sequence and timing of effects leading to death or recovery. These objectives are achieved by means of a comprehensive schedule of animal observations following dosing. These objectives can usually be achieved by treating from one to three groups of three to five mice/sex/group at different doses.

Traditionally, acute toxicity testing of potential new pharmaceutical products is conducted in at least three species, with one being a nonrodent, and by at least two routes of administration, one of which is the intended clinical route. Mice are the most frequently selected rodent species for acute toxicity testing. The choice of routes of administration depends on the intended clinical route and on how much is already known about the oral bioavailability of the test substance. If the intended clinical route is oral, acute testing by oral gavage with a solution or suspension is of primary importance. If other clinical routes are anticipated (e.g., iv or dermal), they represent good secondary routes for acute testing. Ordinarily, at least one parenteral route is used for acute testing, and that route may be intravenous (IV) if the product is soluble in a fairly innocuous vehicle (e.g., water or saline) or ip as a suspension if the product is insoluble in an aqueous (or other innocuous) vehicle. If the intended clinical route is not oral, the oral route is usually selected as a secondary route for acute toxicity testing to provide information relevant to accidental oral ingestion. A rough estimate of oral bioavailability can be based on a comparison of the acute toxicity associated with various doses administered by the oral and parenteral routes. Acute toxicity testing conducted for other purposes is usually more limited in scope. Most regulatory agencies no longer require a full complement of species and routes of administration to render decisions on acute toxicity.

There are a few characteristics of acute toxicity testing that are not common in other toxicity protocols. In a typical repeated dose toxicity study, several groups of animals are treated concurrently with predetermined doses of test substance and a control substance. To reduce animal use in acute toxicity testing, studies that include more than one dose group are usually dosed sequentially, with an interval of at least 24 h between dosing of subsequent groups. This allows the effects of the previous dose to be fully manifested and allows selection of the subsequent dose to provide the highest probability of contributing more useful information. Another unusual aspect of acute toxicity studies is the nutritional status of the animals at dosing. Because some schools believe that the results of acute toxicity testing are more reliable if all animals are in a uniform nutritional state, mice to be dosed orally are often fasted overnight prior to dosing. Fasting allows dose volumes to be higher than in repeated dose studies, and because dosing only occurs on 1 day, dietary stress is considered tolerable. The scientific merits of this practice are debatable, but fasting is "traditional" in oral acute toxicity studies (and in initial human clinical studies). Although the practice of conducting gross necropsies at the end of acute toxicity studies is growing in popularity, this practice rarely yields useful information. The toxicity resulting from acute exposure is usually associated with a biochemical or functional imbalance rather than with a

change in the gross or microscopic architecture of an organ system. Changes observable at gross necropsy are more often associated with repeated dosing at sublethal levels. For similar reasons, microscopic examination of tissue is rarely conducted in acute toxicity studies unless there is some scientific reason to expect it would be useful.

The results of a well-designed acute toxicity study can help to predict likely target organ systems and possible outcome in the event of massive human overexposure, can help in establishing risk categories for EPA or Department of Transportation classification, and can help in dose selection for the initial repeated dose toxicity tests to be conducted. An example of an acute toxicity study design is in Table 2.22.

## Short-Term Toxicity Studies

The objective of short-term or subchronic toxicity studies is to describe and define the toxicity associated with repeated administration of high, but generally survivable doses of a test substance. This may include identification of target organs and systems, definition of the maximum survivable repeated dose, and the highest "clean" or no effect dose. Short-term repeated dose studies also serve as dose range-finding studies for longer-term repeated dose studies.

**TABLE 2.22** Typical Acute Toxicity Study Design for Mice

| | |
|---|---|
| Number of mice/sex/dose group | 3–5 |
| Number of dose groups | 1–3 |
| Number of control groups | None |
| Dosing frequency | Single dose |
| Dosing days | I day |
| Survival checks | Not done (part of Clin. Obs.) |
| Clinical observations | 4 or more on day of treatment, then 1–2 daily |
| Physical examinations | Not done |
| Body weights | Prior to dosing |
| Feed consumption | Not done |
| Number of reversal mice | None |
| Duration of reversal period | Not applicable |
| Blood collection | Not done |
| Hematology parameters | Not done |
| Clinical chemistry parameters | Not done |
| Urine collection | Not done |
| Necropsy | Gross (increasingly, but rarely useful) |
| Tissue collection | Rarely (specific cause only) |

Short-term toxicity studies range in duration of dosing from about 7 to 90 days. Mice typically receive a single, daily dose of the test substance, 7 days/week by the expected clinical route of administration. If the test substance is administered in the diet (or rarely, the drinking water), that admixture is available continuously. Short-term studies usually include three to four groups of mice exposed to different dose levels of the test substance, and an additional group exposed to the carrier to serve as a control for the effects of treatment. Group sizes for these studies are on the order of 5–10 mice/sex/dose. Ideally, dose levels should be selected for these studies such that a few animals die at the highest dose prior to the completion of dosing (to assure exposure to the maximum survivable dose), and all survive at the lowest dose with minimal evidence of toxic effects. The middle dose or doses should be set at approximately equal log increments between the high and low doses. It is important to begin to identify the highest dose level that is free of serious toxic effects to determine whether the test substance is likely to be toxic to humans at the expected therapeutic dose or exposure level.

Parameters monitored in a typical short-term repeated dose study may include daily observations for clinical signs of toxicity and mortality, weekly physical examinations, body weight and feed consumption, and terminal measurement of serum glucose and urea concentrations, serum aspartate aminotransferase, alanine aminotransferase (ALT), and alkaline phosphatase activity. Animals found dead or killed by design are typically submitted for gross necropsy, and selected tissues, such as adrenal gland, bone (sternum, including marrow), brain, heart, kidney, liver, lung, testis, and thymus are collected, weighed (except for bone and lung), and processed for routine microscopic examination by a qualified veterinary pathologist. An example of a short-term toxicity study design is in Table 2.23.

## Chronic Toxicity Studies (26 weeks–2 years)

The objective of chronic, or long-term, toxicity studies is to refine the description of the toxicity associated with long-term administration of high, survivable doses of a test substance. Chronic toxicity studies are more commonly conducted in rats than in mice, but such studies can be conducted in mice, and this discussion describes objectives and practices for conducting such studies. Target organs and systems have usually been identified prior to the conduct of chronic studies, but it is the chronic study that provides the best opportunities to understand the subtle changes associated with