Chemistry Specifications for Chemistry Support Contractors

Division of Translational Toxicology

Animal Studies

## *1. Animal Studies*

Animal studies include, but are not limited to: Toxicokinetic Studies (typically including Preliminary (PTKS) and Definitive (TKS) studies); Absorption, Distribution, Metabolism, Excretion (ADME) Studies (ADMES) and Palatability studies (PALS). Animal studies typically involve single or multiple dosing of an unlabeled or labeled (stable or radioactive isotopes), chemical or test article (referred to as chemical from here on), to rats and/or mice by 1 or more routes e.g., oral gavage, oral feed, dermal, IV, drinking water, etc. TKS, typically conducted using unlabeled chemical, are designed to estimate various toxicokinetic parameters for a chemical and/or a metabolite, e.g., half-life, clearance, etc. and bioavailability. ADMES, typically conducted using a radiolabeled chemical, are designed to evaluate the absorption, distribution, metabolism and excretion properties of a chemical and/or a metabolite. PALS are conducted to determine whether the animal will consume a vehicle at a specified exposure concentration without adverse effects on body weight. The following sections describe requirements that apply to all animal studies performed, regardless of study type. Specific requirements for the PTKS, TKS, ADMES, and PALS Functional Activities are given in Parts 5.2, 5.3, and 5.5, respectively.

1. *General Requirements*

    1. The Contractor shall procure all animals (except B6C3F1/N mice) used in animal studies. B6C3F1/N mice shall be provided by the DTT.
    2. The COR shall provide a study protocol when the assignment is made.
    3. The Contractor shall prepare a study protocol based upon the design provided by the COR.
        1. The Contractor shall submit the protocol to the COR for review.
        2. The Contractor shall obtain approval for the COR-reviewed study protocol from their IACUC, and notify the COR of the approval, prior to ordering animals.
        3. The Contractor's study protocol shall include a milestone schedule that includes dates for some or all of the following at the direction of the COR:
            1. Submission of protocol for IACUC approval
            2. Animal order and expected animal receipt dates

3. Proposed first and last day of dosing
4. Submission of Draft Final report

4. The IACUC-approved study protocol must be posted to the DTT IMS for COR review and approval before the study can commence.

4. At the direction of the COR, the Contractor may be required to ship the samples collected from an animal study to another DTT-designated laboratory for analysis.

*2. Animal Requirements*

1. Animals used in DTT studies shall be humanely treated in accordance with the requirements set forth in Section 1. Part 6. Animal Care and Use Requirements of the DTT Chemistry Specifications.
2. Rats used shall be Harlan Sprague Dawley rats (Hsd:Sprague Dawley® SD®) (HSD) unless specified otherwise and shall be procured by the contractor from an DTT approved laboratory. Mice used shall be B6C3F1/N, unless specified otherwise and shall be provided by the DTT.
3. Animals used in PTKS, TKS and ADMES are typically 10 ± 2 weeks old. For PALS, animals shall be age-matched to the toxicology study in support of which the task is being performed.
4. Animals shall be quarantined for a minimum of 7 days prior to dosing unless COR approval is obtained for a shorter duration. A shortened quarantine period is acceptable for cannulated animals with the approval of the COR.
5. Food and water shall be supplied *ad libitum*, unless otherwise specified in the study protocol. Typical feed types used in DTT studies are: NTP-2000, NIH-07, or Lab Diet 5K96, and shall be specified in the DTT study protocol.
6. Animals shall be randomly assigned to each time point and/or exposure group. Body weights of animals shall be recorded on the first day of dosing and at sacrifice, unless specified otherwise.
7. Animals shall be euthanized via exposure to 100% $CO_2$. Death shall be confirmed by a secondary method (e.g. exsanguination).

*3. Formulation Requirements*

*1. Preferred Dose Vehicles*

1. Gavage (solutions): deionized water

2. Gavage (suspensions): 0.5% methylcellulose in deionized water
3. Feed:
    1. Irradiated NTP-2000 meal (toxicity or carcinogenicity studies)
    2. Irradiated NIH-07 (perinatal, reproductive or developmental studies)
    3. Irradiated Lab Diet 5K96 (low phytoestrogen, reproductive studies)
4. Drinking water
    1. Tap water
    2. Purified water (study-specific)
5. Dermal:
    1. 95% ethanol
    2. Acetone
6. IV:
    1. 0.9% saline
    2. Cremophor EL™:ethanol:water, 1:1:8

*2. Typical Dose Volumes:*

1. IV: 2 mL/kg for rats and 4 mL/kg for mice
2. Gavage: 5 mL/kg for rats and 10 mL/kg for mice
3. Dermal: 0.5 mL/kg for rats and 2.0 mL/kg for mice

*4. Exposure Requirements*

The following requirements are typical, alternate approaches must be approved by the COR. For dermal studies, approximately 24h prior to dosing, animals' backs and shoulders shall be carefully clipped to remove the fur. The clipped area shall be inspected for cuts and nicks. Any animals with damaged skin in the area intended for dosing shall be replaced. The formulation shall be applied uniformly to a fixed standard area of skin in the dorsal (e.g. interscapular) region for both rats and mice. The dose site may be protected or unprotected from grooming, and shall be specified in the DTT study protocol. This area is to be the same size and location for each animal of a given species. The application site typically is 1 x 1 cm for mice and 2 x 2 cm for rats.

*1. Single Exposure Requirements*

1. IV route: Groups of male and/or female rats and/or mice shall be dosed intravenously with a formulated chemical at 1 dose concentration.

2. Gavage route: Groups of male and/or female rats and/or mice shall be dosed at each of up to 3 dose concentrations via oral gavage.
3. Dermal route: Groups of male and/or female rats and/or mice shall have test article applied dermally at each of up to 3 dose concentrations..

2. *Multiple Exposure Requirements*

1. All studies: Daily food and water consumption shall be measured in all multiple dosing studies, unless specified by the COR.
2. Gavage Route: Groups of male and/or female rats and/or mice shall be dosed via oral gavage at each of up to 3 dose concentrations of test article for up to 14 days. Animals shall be dosed on the morning of the day after the last full day of dosing, following which, biological samples shall be collected as specified in the study protocol.
3. Dermal Route: Groups of male and/or female rats and/or mice shall have test article applied dermally at each of up to 3 dose concentrations for up to 14 days. Test article shall be applied to animals on the morning of the day after the last full day of dosing, following which, biological samples shall be collected as specified in the study protocol.
4. Feed Route: Groups of male and/or female rats and/or mice shall be exposed to dosed feed ad libitum at each of up to 3 exposure concentrations of test article for up to 14 days. PALS require an additional concomitant control group. On the morning of the day after the last full day of exposure, biological samples shall be collected as specified in the study protocol.
5. Drinking Water Route: Groups of male and/or female rats and/or mice shall be exposed to dosed water ad libitum at each of up to 3 exposure concentrations of test article for up to 14 days. PALS require an additional concomitant control group. On the morning of the day after the last full day of exposure, biological samples shall be collected as specified in the study protocol.

5. Biological Sample Collection

1. Biological samples shall be collected as specified in the study protocol.

2. Following appropriate anesthesia, biological samples (e.g., blood, tissues, excreta) shall be collected from 1 to 3 animals per endpoint and depending on the study type.
3. If plasma is to be analyzed $K_3$EDTA shall be used as an anticoagulant unless otherwise directed by the COR.
4. Biological samples collected during studies shall be stored in appropriate containers at ≤ –70ºC unless otherwise specified in the study protocol.

## *2. Preliminary Toxicokinetic Study (PTKS)*

A PTKS involves single or multiple dosing of a chemical, which may be labeled or unlabeled, in a single animal per time point, unless otherwise specified in the DTT study protocol. PTKS studies are conducted to help inform the design of the TKS.

*1. Requirements*

1. As directed by the COR, the Contractor shall conduct a PTKS via an intravenous (IV) and/or other appropriate route(s) to administer the chemical.
2. Exposure via IV typically requires 1 exposure concentration. Exposures via other routes may require up to 2 doses/exposure concentrations.
3. For each group, chemical shall be administered to 1 animal for each time point (typically 10) at which blood and/or tissues are to be collected, unless specified otherwise in the study protocol.
    1. At each time point, as much blood as possible shall be collected from the vena cava or other appropriate site.
    2. Prior to dose administration, a sample of blood shall be collected to serve as a pre-dose sample (or zero time point).
4. Confirmatory dose formulation analyses shall not be conducted in a PTKS.
5. Concentrations of chemical(s), metabolites, and/or other appropriate marker compound(s) in plasma and/or tissues collected from PTKS shall be determined in a separate assignment, typically biosample method development and analysis (Part 4.1. BMDA).
6. The contractor, using non-compartmental methods, shall evaluate plasma and/or tissue concentration vs. time data.

7. The QC'd preliminary results shall be tabulated in accordance with interim data requirements found in Section 4. Reporting Requirements, and posted to the DTT IMS upon completion of the analyses within a timeframe specified by the COR.
8. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

## *3. Definitive Toxicokinetic Study (TKS)*

A TKS involves single or multiple dosing of a chemical, which may be labeled or unlabeled, in a minimum of 3 animals per time point, unless otherwise specified in the DTT study protocol. TKS studies are conducted to establish basic toxicokinetic parameters and determine bioavailability.

*1. Requirements*

1. As directed by the COR, the Contractor shall conduct a TKS via an intravenous (IV) and other appropriate route(s) to administer the test chemical.
2. SOPs pertaining to the collection and analysis of biological samples obtained from the study shall be posted to the DTT IMS along with the study protocol and shall be included in the toxicokinetic study record.
3. Exposure via IV typically requires 1 exposure concentration. Exposures via other routes typically require 3 doses/exposure concentrations.
4. Typically, a minimum of 10 time points are specified for each dose concentration-dose route combination.
5. For each group, chemical shall be administered to a sufficient number of animals for each time point at which blood and/or tissues are to be collected, such that usable data is obtained from a minimum of 3 animals per time point.
    1. Prior to dose administration, a sample of blood shall be collected to serve as pre-dose time point.
    2. To minimize animal usage for rat studies, blood may be collected from each animal at 2 or more time points and for mouse studies, at 2 time points, at least 2 hours apart. At the direction of the COR, cannulated animals may be used to collect a complete time course.

3. Total blood sample volume taken at interim bleeds shall not exceed 2.0% of body weight for rats and 2.5% of body weight for mice.
4. Tissues shall only be collected at the second time point.
    1. Tissue collection procedures shall be specified in the study protocol.

6. Confirmatory dose formulation analyses shall be conducted in all TKS using a validated analytical method in a separate assignment, typically formulation preparation and analysis (Part 3.6. FPA).
7. Concentrations of chemical, metabolites, and/or other appropriate marker compound(s) in plasma and tissues collected from TKS shall be determined in a separate assignment, using a validated method, typically biosample analysis (Part 4.3. BSA).
8. The contractor, using non-compartmental methods, shall evaluate plasma and/or tissue concentration time course data collected for each exposure group. When appropriate, compartmental models may be used to supplement the non-compartmental approach.
9. The QC'd preliminary results of the analysis shall be tabulated in accordance with interim data requirements found in Section 4. Reporting Requirements, and posted to the DTT IMS upon completion of the analyses within a timeframe specified by the COR.
10. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

## *4. Absorption, Distribution, Metabolism and Excretion Study (ADMES)*

An ADMES involves single or multiple dosing of a chemical. ADMES are conducted to investigate absorption, distribution, metabolism, and excretion and determine mass balance following exposure to radiolabelled chemical, typically [$^{14}$C], unless specified otherwise in the study protocol. The radiochemical purity shall be ≥ 95% unless approved by the COR.

*2. <u>Requirements</u>*

1. As directed by the COR, the Contractor shall conduct an ADMES via an intravenous (IV) and other appropriate route(s) to administer the test chemical.

2. Number of animal groups required for ADMES depend on the, route of exposure, species, and sex and shall be specified in the DTT study protocol.
3. For each group, chemical shall be administered to up to 3 animals per group.
4. Radiochemical purity shall be determined by the Contractor prior to the start and during an ADMES.
5. Formulation development and analysis methods and formulation stability shall be investigated by the Contractor prior to study start.
    1. Method development shall use unlabeled chemical, unless directed by the COR.
    2. Previously developed analytical methods and/or methods developed by the Contractor shall be qualified, but not validated, using the radiolabeled chemical.
6. To prepare dose formulations for an ADMES, radiolabelled chemical shall be diluted with unlabeled chemical such that the total radioactivity used shall be kept constant, not to exceed 50 µCi/rat and 10 µCi/mouse unless specified by the COR.
7. Radiochemical concentration in formulations shall be checked prior to and after dosing. The exact administered dose and the radioactivity shall be reported.
8. Animals shall be acclimatized individually in metabolism cages that allow for the separate collection of excreta approximately 24h prior to dose administration.
    1. Following administration, animals shall be returned to metabolism cages. Animals shall continue to be individually housed in metabolism cages until the study is completed.
9. Dermal studies shall be conducted with the application site covered, unless specified otherwise in the DTT study protocol. A foam appliance (rats) or a metal capsule (mice) shall be used to cover the dermal dose site.
10. Study duration following first or last dose administration shall be 72h, unless specified otherwise in the DTT study protocol.

11. Excreta, blood, and tissues shall be collected; weights (or volumes) shall be recorded for each collection period (see below), and stored separately per time point.
    1. Typical collection period (h) for excreta (e.g., urine, feces, exhaled volatile organics (VOCs), and $CO_2$) are: urine, 0-8, 8-24, and every 24h intervals thereafter; feces, 0-8, 8-24, and every 24h intervals thereafter; VOCs and $CO_2$, 0-4, 4-8, 8-12,12-24, and every 24h intervals thereafter.
        1. Urine shall be collected separately from each animal into receivers cooled over dry ice. Urine from the bladder at sacrifice shall be added to the last urine collection. Following each collection period the cage shall be rinsed with water (with last rinse with ethanol) and rinsates shall be collected separately from urine.
        2. Volatile compounds shall be collected, at or below 0°C during collection, by passing the air from the metabolism cage first through two traps containing isopropanol to trap VOCs and then through two traps containing 1N NaOH to trap $CO_2$.
            1. Based on preliminary data, if less than 1% of the total administered radioactivity is found to be in the traps, the collection of expired air shall terminate in subsequent studies following approval of the COR.
    2. Control samples of excreta shall be collected during the acclimation period to determine background radioactivity.
12. At study termination blood (highest volume possible and not less than 4 mL in rats and 0.5 mL in mice) shall be collected by cardiac puncture using $K_3$-EDTA as an anticoagulant. Plasma shall be isolated from a fraction of blood.
13. Tissues and glands and organs (in their entirety) shall be collected and weights shall be recorded. Tissues collected typically include, but are not limited to, adipose (perirenal), skeletal muscle (hind leg), akin (abdominal), liver, lung, kidneys, brain, spleen, heart, pancreas, thyroid, urinary balder, testes, uterus (with fallopian tubes), ovaries, small intestine, large intestine, stomach, cecum, dose sire skin (in dermal studies).
    1. Contents from gastrointestinal tract (GI) (small intestine, large intestine, stomach and cecum) shall be removed, combined and weighed and stored separately from the GI tract tissues.

2. GI tissues shall be rinsed with deionized water unless directed otherwise by the COR and the rinsate shall be combined with the contents.
3. The carcasses remaining after tissue collection shall be saved for possible further sampling or solubilization of whole carcass for mass balance determinations.
4. In dermal studies, the dose site skin shall be excised and rinsed with a suitable solvent. The rinsate and the skin shall be saved as well as the dermal appliance used to protect the dose site when applicable.

14. The Contractor shall analyze all samples in triplicate for total radioactivity using Liquid Scintillation Spectroscopy (LSS) either directly (after dissolution in a scintillation cocktail), after oxidation in a sample oxidizer, or after solubilization.
15. The Contractor shall perform metabolic profiling and metabolite identification in urine or other matrices as directed by the COR.
    1. Metabolite profiles shall be obtained using HPLC with radioactive detection.
    2. Profiling shall be done before (all groups) and after enzyme (e.g., glucoronidase, sulfatase, acylase) deconjugation (selected groups) as directed by the COR.
    3. Metabolite identification shall be done using mass spectrometry, NMR spectroscopy or other appropriate analytical techniques in urine (or other matrices if directed by the COR) from one dose group (typically highest male rat group).
        1. If urinary HPLC radioprofiles show differences between doses, routes, species, or sex, the metabolite identification shall be done for other selected groups following approval by the COR.
16. The QC'd preliminary results of the analysis shall be tabulated in accordance with interim data requirements found in Section 4. Reporting Requirements, and posted to the DTT IMS upon completion of the analyses within a timeframe specified by the COR.
17. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

## *5. Palatability Study (PALS)*

1. At the direction of the COR the Contractor shall conduct a palatability study, which typically consists of 2 rodent species and 2 sexes dosed via drinking water or feed until it is apparent that the formulation is not palatable, or 14 days, whichever comes first.
    1. Palatability studies typically consist of 3 dosed groups plus controls. Dosed groups typically consist of 5 or fewer animals, housed singly.
    2. The Contractor shall formulate the doses using the best available methods under a Formulation Preparation assignment. Formulation analysis is not typically required. When dose analysis is required by the COR:
        1. The Contractor shall perform a Formulation Development (Part 3.1) assignment to develop, but not validate a dose analysis method.
        2. The Contractor shall perform a Formulation Preparation, Analysis and Shipment (Part 3.7) assignment to analyze the formulations.
    3. Food and water consumption shall be measured daily or as specified in the protocol during the course of the study. Other endpoints may be monitored at the direction of the COR.
    4. Animal body weights shall be determined during the course of the study on days 1, 7, and 14 or other days specified by the COR.
    5. Notation shall be made if animal feces, bedding, water, or urine are present in feed, or if there is evidence of scattered feed or playing with water.
2. The preliminary results of the study metrics shall be tabulated and posted to the DTT IMS immediately upon completion of the analyses or observations, or at least weekly.
3. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.