Chapter 6. Functional Areas

Specifications for the Conduct of Chemistry Activities for Toxicology Research Performed by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences

6. Functional Areas

Bradley J. Collins[1], Suramya Waidyanatha[1] (Editors)

[1]Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Last Updated: May 2026

6.1. General

All communication shall be conducted, and information shall be provided using the Division of Translational Toxicology (DTT) Project Management System (PMS).

Proposed plan and estimated cost for an assigned activity shall be provided in the PMS for the contracting officer's representative (COR) approval. The contractor shall not commence any activity prior to COR approval.

A milestone schedule that includes dates for the following shall be provided by the contractor for each assignment, as applicable, at the direction of the COR:

- Commencement of lab work
- Completion of lab work
- Completion of draft final report
- Commencement of quality control (QC) review
- Commencement of quality assurance review
- Submission of draft final report

All acceptance criteria must be met as specified under the specific functional activity. When analysis results in values outside the acceptance criteria, the contractor shall document the issue and immediately notify the COR.

The contractor shall report the results for each task performed using the requirements given in Chapter 7. Reporting Requirements.

6.2. Logistics and Handling

6.2.1. Chemical Procurement (CP)

As directed by the COR, the contractor shall identify sources and availability of required test articles.

The contractor shall check the DTT[1] chemical inventory for each chemistry support contract to determine whether the test article(s) are available prior to identifying commercial sources.

[1]Refer to Glossary of Terms.

The contractor shall post the sourcing and quotation information, including the supplier certificate of analysis (COA) as it is received.

As directed by the COR, the contractor shall procure the necessary quantities.

The contractor shall post the expected delivery date as soon as it is known.

The contractor shall visually examine the material on receipt and record the condition of the shipping container(s) and contents, the shipping documents (e.g., COA, safety data sheet [SDS]), and the lot number. If an SDS is not included in the shipment, the contractor shall request an SDS from the supplier. If an SDS cannot be obtained from the supplier, the contractor shall create one. The record shall include a photograph of the shipping container and contents, as well as the shipped material. A photo of the test article is not necessary unless required by the COR. Any photographs taken of the test article shall include color and size scales.

For each test article received, the contractor shall enter the name and/or other identifiers (e.g., distributed structure-searchable toxicity substance identifier [DTXSID], chemical class [e.g., flame retardant, per- and polyfluoroalkyl substances (PFAS), ionic liquid], Chemical Abstracts Service Registry Number® (CASRN), source, amount received, lot number, and receipt date), along with other appropriate identifying information, into an electronic chemical inventory system.

The contractor shall store the received material under the manufacturer's recommended conditions until on-receipt handling is complete unless otherwise directed by the COR. The contractor shall consult the COR regarding on-receipt storage of complex mixtures (e.g., botanical materials).

6.2.2. Multiple Chemical Procurement (MCP)

The contractor shall procure and/or receive up to 100 test articles (typically <100 g each) to support toxicity screening activities (e.g., medium throughput screening activities in cell based or small animals models (e.g., Zebrafish)) . The contractor shall identify sources and availability for lists of test articles provided by the COR.

As part of sourcing, and prior to procurement from a commercial source, the contractor shall check the chemical inventory to determine whether the chemical(s) are available.

The contractor shall post the sourcing and quotation information to the DTT PMS as it is received. The contractor shall post expected delivery date(s) for each chemical—and the estimated time to receive all chemicals ordered—to the DTT PMS as soon as they are known.

The contractor shall record the source and amounts received in the toxicity screening chemical inventory and assign and/or record chemical identification codes, including the CASRN and DTXSID number.

When all chemicals have been received, and the list is considered complete, the contractor shall provide the list with all associated chemical information (e.g., source, lot number, CASRN, DTXSID) to the COR.

- Refer to the EPA CompTox database[2] to obtain DTXSID numbers. When a received chemical is not found in this database, the contractor shall notify the COR, who shall request a DTXSID from EPA.

The contractor shall examine the received material and record the condition of the material, including the shipping container(s) and contents, the shipped material (bulk chemical, test article, etc.), and the lot number(s).

If the shipping container is damaged or leaking upon receipt, the contractor shall immediately notify the COR, who may direct that photographs of the received material be taken. A description of the issue shall be immediately posted to the DTT PMS and if photographs are taken, they shall be posted to the DTT PMS as soon as they are available.

6.2.3. Chemical Handling (CH)

As directed by the COR, the contractor shall perform some or all of the following activities:

- A chemical handling plan describing any proposed handling to be performed on the test article shall be posted to the DTT PMS for approval by the COR.
- Upon receipt of a test article, the contractor shall perform the following:
  - If not already performed under CP, visually examine the received material, including the shipping documents (COA, SDS, etc.), and record the condition of the shipment, including the shipping container(s) and contents and the test article. If an SDS is not included in the shipment, the contractor shall request an SDS from the supplier or prepare one if not available from the supplier. If the shipping container has not been photographed, a photograph of the container shall be taken.
  - Photograph the test article. Manually mix the received test article prior to removing an aliquot to photograph. Photographs of the test article shall be taken against a color spectrum reference card that also includes a scale to estimate particle size. Photographs shall be posted to the DTT PMS as soon as they are available.
  - Record the appearance of the test material (e.g., physical state, color).
  - Remove a single 100 g archive sample, ten 5 g analytical samples, and sufficient additional aliquots to cover test article characterization and formulation and biosample analysis method development needs. The COR may specify the amounts to be removed in some circumstances. If the test article is to be handled (e.g., homogenization, particle size reduction), aliquoting should be taken following final handling.
  - Unless specific properties of the test article require other storage conditions:
    - The archive sample shall be stored at ≤ −20°C.
    - The analytical (frozen reference) samples shall be stored at approximately −20°C unless test article properties prevent frozen storage.
  - All archive and analytical samples shall be logged into an electronic inventory system.

[2] https://comptox.epa.gov/dashboard

- If handling of test article is required, as directed by the COR, the contractor shall perform one or more of the following activities on a test article:
  - Homogenize multiple containers of the test article (in specific circumstances, the COR may require that archive and analytical samples be removed before and after homogenization).
  - Reduce the particle size to an appropriate size, as necessary (typically ≤100 μm).
  - As necessary, aliquot and/or repackage the material into different containers.
  - Handled test material shall be assigned lot numbers different from the original lot number and entered into an electronic inventory system including all relevant information (e.g., chemical name, CASRN, lot number).

The contractor shall complete the handling of newly procured test articles within 2 weeks of receipt.

6.2.4. Multiple Chemical Handling (MCH)

As directed by the COR, the contractor shall perform some or all of the following activities for a set of test articles procured under one or more MCP assignments in support of toxicity screening activities.

- Combine and/or homogenize test articles received in multiple packages.
- Determine the solubility of each chemical at concentrations up to 100 mM in a solvent, typically DMSO,[3] or as directed by the COR.
- Prepare solutions of each test article in the set, typically 10 mL at 100 mM or the maximum soluble concentration, if <100 mM in DMSO, or a solvent and volume designated by the COR.
- Dispense small volumes (typically 0.300 or 1.00 mL) of test article solutions into screw-capped tubes contained in 96- or 384-tube racks (plates).[4] At the direction of the COR, multiple copies of each rack (plate) or multiple racks/plates may be prepared.

When plates are prepared, the contractor shall create a plate map for each plate produced, which consists of a spreadsheet (Excel or equivalent) containing specific information about each chemical on the plate. An example plate map is given in Appendix B.

- The contractor shall record the plate number of each plate on its corresponding plate map.
- The contractor shall examine the plated chemicals and record the condition of the plate(s) prior to shipment.

[3]DMSO: ACS spectrophotometric grade, Sigma-Aldrich Product No. 154938, or equivalent.
[4]Micronic 96-Q1, 96-1 (0.5 mL), or 96-4 (1.4 mL), Krystal™ 384 Well Microplates or equivalent or as directed by the COR.

6.2.5. Chemical Storage (CS)

As directed by the COR, the contractor shall store at its facility chemicals, test articles, formulations, and/or vehicles received from DTT-designated laboratories or investigators.

The contractor shall examine the material on receipt, including the shipping documents (COA, SDS, etc.), and record the condition of the material, including the shipping container(s) and contents and the shipped material (test article, formulation, and/or vehicles). The record shall include a photograph of the shipping container and contents and, at the direction of the COR, the shipped material, including a color scale.

For each test article received, the contractor shall enter the chemical name, CASRN, and/or other identifier (e.g., DTXSID, chemical class [e.g., flame retardant, PFAS, ionic liquid]), source, amount received, lot number, receipt date, and study number as appropriate into an electronic inventory system.

6.2.6. Shipment (SHIP)

As directed by the COR, the contractor shall aliquot, package, and/or ship test articles, including those supporting toxicity screening activities, formulations, vehicles, solutions, and/or biological samples to DTT-designated laboratories or investigators.

- The contractor shall confirm shipping details with the recipient prior to shipment.
- At least 24 hours prior to shipment, the contractor shall notify the recipient of the ship date and expected delivery date.
- The contractor shall ensure materials are shipped under conditions that will ensure their stability.

All applicable documentation including the SDS, COA, and/or other sample information as directed by the COR, shall be provided with all materials shipped by the contractor to a DTT-designated laboratory. If an SDS is required and is not available from the manufacturer, the contractor shall develop one to accompany any shipment.

When the purpose of the shipment is to transfer a bulk test article to a toxicology laboratory in support of a study, the contractor shall include a 5 g frozen reference sample of the bulk test article with the shipment. A smaller amount of frozen reference material can be sent or material may be excluded from a shipment at the direction of the COR.

As directed by the COR, the contractor shall arrange for the transport of test articles, including toxicity screening test articles, formulations, vehicles, solutions, and/or biological samples from DTT-designated laboratories or investigators to its facility.

All shipments shall conform to the requirements given in Chapter 3. Health and Safety and all applicable local, state, and federal regulations.

For toxicity screening support activities, the following additions will be included.

COR will provide the list of chemicals or solutions to be shipped including at a minimum the following information.

- CLID

- Chemical name
- CASRN
- Amount
- Requestor
- Requestor branch/department
- Recipient
- Recipient company
- Recipient company department
- Shipping date
- Program of work
- Keywords
- Assay
- Contributor

Prior to shipment of chemicals, the contractor shall prepare a file to include the following information:

- Blind-code (if applicable)
- Molecular weight
- Chemical name
- DTXSID
- Amount
- Amount unit
- Supplier
- Lot number
- Concentration
- Concentration units
- COA purity (%)
- Determined purity (%)
- Storage condition/suggestions (codes)
- Plate position
- Additional information
- Simplified Molecular Input Line Entry System (SMILES) codes

Metadata shall be included beginning in Row 1, Column A, Tab 1 with the Chemical List headers in Row 17, Column A. Changes to the file format must be approved by the COR.

6.2.7. Toxicity Screening Chemical Inventory Maintenance

The contractor shall maintain an inventory of all chemicals procured and/or received to support DTT toxicity screening research and aliquots, solutions, or other formulations derived from them.

All materials contained in the inventory shall be maintained under appropriate storage conditions.

Identity and purity of test articles and stability of solutions in the inventory shall be reassessed prior to use when the following conditions are met:

- Upon chemical receipt and/or solution preparation
- The purity of the test article has not been assessed during the preceding 24 months
- The solution was prepared more than 12 months ago, or the test article is known or suspected to be unstable in the solvent
- Other intervals defined by the COR

The contractor shall create and maintain a searchable database of all test articles procured and/or used in medium to high-throughput DTT studies and all aliquots, solutions, and formulations derived from them.

The database shall track, at minimum, the following metadata:

- Chemical name
- CASRN
- DTXSID
- Chemical List Identification number (CLID, when applicable)
- Source and lot
- Receipt date
- Amount on hand
- Last purity analysis date and determined purity
- Chemical class information (one chemical may belong to multiple classes)
- Use tags (e.g., pesticide, flame retardant) or other tags specified by the COR
- Other information that may from time to time be specified by the COR.

The database shall be updated whenever a new test article is received, a new lot or additional amount of an existing lot is received, and/or when an aliquot of a test article is shipped from the inventory. The inventory shall be updated such that the current availability of all chemicals is accurately displayed. The inventory shall also be updated when new information (e.g., chemical classification, chemical use) becomes available.

The inventory shall be searchable. Search fields shall include, but are not limited to, chemical name, CASRN, DTXSID, chemical class/tags.

The database shall be accessible to DTT staff via user-specific login. The COR must approve all requests for new login credentials.

6.2.8. Protocol Development (PD)

At the direction of the COR, the contractor shall develop and apply protocols for use by DTT-designated laboratories and/or investigators for one or more of the following analysis types:

- Formulation Preparation
- Formulation Analysis Method Qualification and/or Validation
- Formulation Analysis
- Test Article Handling and Analysis
- Special Handling

Formulation Preparation Protocol

The contractor shall develop a formulation mixing protocol for a designated test article in a specified vehicle based on the procedures described in a related formulation development (FD) assignment.

The mixing protocol shall describe a step-by-step procedure required to prepare a uniform formulation at the volume or weight prepared for the FD assignment.

The mixing protocol shall include long-term storage requirements determined in a related formulation stability evaluation.

Formulation Analysis Method Validation Protocol

The contractor shall develop a formulation analysis method qualification and/or validation protocol for a designated test article in a specified vehicle based on the procedures described in a related FD assignment.

The formulation analysis validation protocol shall describe a step-by-step procedure required to perform a full method validation, excluding method verification.

Formulation Analysis Protocol

The contractor shall develop a protocol describing the analysis procedure for a designated test article, formulated in a specified vehicle.

The procedure shall be based on the analysis method described in a related FD assignment and the formulation analysis requirements found in the Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences, March 2023 (NIH 2023). For specific studies (e.g., 5-day studies), the COR may indicate alternate specifications to be followed.

Test Article Handling and Analysis Protocol

At the direction of the COR, the contractor shall develop protocols for the receipt, storage, and initial and subsequent analyses of the test article, including any special handling requirements.

Receipt and storage requirements described in the protocol shall be derived from comprehensive chemical analysis (CCA) functional activity.

Procedures to establish identity upon receipt shall employ appropriate techniques derived from the CCA.

Analysis methods used to determine purity—upon receipt or during a study—shall be based on the validated dose analysis method for the test article and shall include an internal standard (IS).

- The protocol purity method shall employ calculation of a relative response factor (RRF) for replicate analyses of the test article and a frozen reference sample of the same lot.
- A mean and 95% confidence interval (CI) shall be calculated for the frozen reference sample RRF.
- The RRF for the test article shall be compared to the RRF for the frozen reference.
- When the RRF for the test article lies outside the 95% CI of the frozen reference standard or when impurities that were not present in the on-receipt analysis are observed, the testing lab shall be instructed to contact the study director.

Special Handling Protocols

At the direction of the COR, the contractor shall develop handling protocols for test articles or a list of test articles with special handling requirements (e.g., biological sample collection for volatile analytes).

Protocol contents shall be tailored to the specific requirements of the chemicals covered by the protocol.

6.3. Characterization

6.3.1. General Requirements

For all characterization activities, measured physical parameters, spectra, and chromatograms shall be compared to a reference material (e.g., certified reference material) or standard literature reference, when available.

6.3.2. Comprehensive Chemical Analyses (CCA)

General Requirements

Determine the identity and purity of a specified test article.

A characterization plan shall be developed and posted to the DTT PMS[5] for approval by the COR. The characterization plan shall describe the general analytical approach based on the test article type and all the steps to be performed for the characterization of the test article, including, but not limited to, the following:

[5]Refer to Glossary of Terms.

- A list of the proposed analyses to be performed, including analytical techniques and method parameters, if known.
- Literature reference(s) that serve as a starting point for the characterization, when available.

Analysis Requirements

The contractor shall determine the following, as appropriate, for the test article to establish its identity and purity unless otherwise directed by the COR.

Organics

Determine the unequivocal identity using two or more spectrometric techniques (including, but not limited to, infrared [IR] spectrophotometry, proton and C-13 nuclear magnetic resonance [NMR] spectrometry, and mass spectrometry [MS]). NMR spectrometry shall include other (e.g., 2D NMR) analyses when appropriate.

Determine the purity:

- The contractor shall use at least two different analytical techniques, such as:
  - One chromatographic system and quantitative NMR (qNMR).
  - Two chromatographic systems with different separation mechanisms (e.g., gas chromatography [GC] and high-performance liquid chromatography [HPLC], normal and reverse phase HPLC).
- Purity analysis methods shall be maximized for separation of all components. Nonselective detectors shall be used (e.g., flame ionization detection [FID], charged aerosol detection).
- The contractor shall determine the water content of the test article using an appropriate analytical method.
- As directed by the COR, the contractor shall conduct other analyses (e.g., elemental composition (e.g., C, H, O), thermogravimetric, differential scanning calorimetry).
- As directed by the COR, the contractor shall determine the volatile content of all solids using a literature method.
- Determine the impurity profile and identify impurities at concentrations ≥1.0%.
- In some instances, the COR may require the contractor to identify impurities at concentrations <1.0%. The COR may direct that any impurities be quantitated against reference standards when they are available.
- Determine the physical characteristics (e.g., melting point, optical rotation).
- Perform compendial analyses when appropriate (e.g., pesticide residues, metals content, nutritional analysis).
- The contractor shall report the log P value when available in the literature. If the log P value is not available in the literature, as directed by the COR, the contractor shall determine the log P value using a literature method.

Determine the stability (accelerated stability) of the test article after storage at −20°C, 5°C, ambient temperature, and 60°C ± 2°C for 2 weeks.

Inorganics

Determine the unequivocal identity using elemental analysis, microscopy, and spectrometric techniques (e.g., scanning electron microscopy [SEM], transmission electron microscopy [TEM], x-ray diffraction [XRD], and inductively coupled plasma-mass spectrometry [ICP-MS]) as appropriate.

Determine the purity:

- The contractor shall use two different analytical techniques when feasible (e.g., ion chromatography, ICP-MS, XRD).
- Determine the water content of the test article using an appropriate analytical method.
- As directed by the COR, the contractor shall determine the volatile content of all solids using a literature method.
- Determine the impurity profile and identify impurities at concentrations ≥1.0%.
- In some instances, the COR may require the contractor to identify impurities at concentrations <1.0%. The COR may direct that impurities be quantitated against reference standards when they are available.
- Determine the physical characteristics (e.g., particle size).
- Perform compendial analyses when appropriate.
- Determine the stability (accelerated stability) of bulk chemicals after storage at −20°C, 5°C, ambient temperature, and 60°C ± 2°C for 2 weeks.

Complex Mixtures

- Complex mixtures (e.g., botanical dietary supplements, nanomaterials, asbestos, organometallics) shall be characterized to the same extent as organics and inorganics.
- Characterization may require the use of techniques other than those found in the Organics or Inorganics sections above.
- Examples of requirements for complex chemical mixtures are given below:
    - **Botanical Dietary Supplements:** In addition to the requirements given in the Organics and Inorganics sections above, the contractor shall determine one or more of the following, at the direction of the COR:
        - Whether the test article comes from the correct plant species. This analysis may involve, but is not limited to, authentication using high-performance thin-layer chromatography and/or DNA barcoding techniques.
        - Phytochemical profile using nontargeted analysis (NTA) methods and identification of botanical-specific constituents (e.g., sennosides in Senna).
        - Constituent concentration using qualified and/or validated analytical methods.
        - Nutritional content (e.g., carbohydrates, protein, fat) and contaminants (e.g., heavy metals, pesticide residues, mycotoxins, bacterial load).
    - **Nanomaterials:** In addition to the requirements given in the Organics and Inorganics sections above, the contractor shall perform one or more of the following analyses of nanomaterials, at the direction of the COR. See National

Cancer Institute, Nanotechnology Characterization Laboratory, Assay Cascade Protocols[6] for more information.

- Size determination, including diameter and length (for fibers). Size determination may involve, but is not limited to, the use of dynamic light scattering, atomic force microscopy, SEM, or TEM.
- The contractor shall determine the chemical composition of nanomaterials, including metal content.
- The contractor shall determine surface characteristics (e.g., Zeta potential, electrolytic conductivity of nanoparticle suspensions, pH of nanoparticle suspension), using one or more techniques, at the direction of the COR.

6.3.3. Chemical Identity and Purity Screen (CIPS)

Analysis Requirements

At the direction of the COR, the contractor shall determine the identity and estimate the purity of the test article.

- Unless directed by the COR, the analysis shall consist of a single analytical system (e.g., qNMR, liquid chromatography-mass spectrometry [LC-MS]) selected to unambiguously and simultaneously identify the test article and estimate the test article's purity.
- When applicable, analysis conditions shall be maximized for the separation of all components to allow purity determination.
- The system selected for each analysis shall be the simplest and most cost-effective system that can successfully meet the analytical criteria.

6.3.4. Multiple Chemical Identity and Purity Screen (MIPS)

At the direction of the COR, the contractor shall determine the identity and estimate the purity of a set of test articles procured under one or more MCP assignments or of a list of test articles provided by the COR. A MIPS may be applied to any list/set of test articles as a purity and identity check upon receipt or in lieu of more extensive characterization.

Unless directed by the COR, the analysis shall consist of a single analytical system to unambiguously identify the test article and estimate its purity simultaneously. Systems selected for identity and purity analyses shall be the simplest and most cost-effective that can successfully meet the analysis criteria

- The system selected for each analysis shall be the simplest and most cost-effective system that can successfully meet the analysis criteria.
- When a chromatographic method is used, it shall employ a temperature or mobile-phase gradient that achieves satisfactory, but not necessarily baseline, separation of all chemical components.

[6] https://www.cancer.gov/nano/research/ncl/protocols-capabilities

- When the set of chemicals or test articles is part of a chemical class, the analytical method developed shall be such that it can be applied to all other chemicals or test articles in the class with minimal optimization.

Purity of chemicals selected for toxicity screening evaluation must be ≥95% unless the COR approves a different target value.

All measured physical parameters, spectra, and chromatograms shall be compared to a known standard or standard literature reference value, when available.

Typical analytical systems, which may be used for MIPS analyses, include the following:

- Organic chemicals
  - qNMR
  - High-performance liquid chromatography-mass spectrometry (HPLC-MS), including ultra-high-performance liquid chromatography (UPLC) and HRMS
  - GC-MS
- Inorganic chemicals
  - Inductively coupled plasma-mass spectrometry (ICP-MS) or inductively coupled plasma-atomic emission spectroscopy (ICP-AES)

6.3.5. Chemical Reanalysis (CRA)

At the direction of the COR, the contractor shall perform the following analyses on a test article:

- The contractor shall perform a CRA of test articles submitted by DTT testing laboratories within 1 week of receipt. The COR will provide a schedule describing the expected shipment and analysis dates for CRA samples.
- Prior to shipment of bulk chemicals to a DTT testing laboratory or the use of test articles to prepare formulations, the contractor shall perform a CRA of test articles that have been stored for over 6 months since the previous analysis (e.g., CCA, CRA).
- Visual Comparison: The contractor shall compare the appearance of both the test article and the frozen reference standard to a photograph taken at chemical handling.
- At the direction of the COR, the contractor shall perform the following analyses:
  - The contractor shall use previously developed methods, as described under 6.2.8. Protocol Development (PD) to determine the purity of the test article relative to a frozen reference standard of the same lot or to another reference standard (e.g., standard reference material [SRM]) specified by the COR. When the CRA requires the use of a substance-specific standard operating procedure (SOP), it must be in place prior to its use.
  - When the test article is a complex mixture (e.g., botanical dietary supplement), the COR may direct the contractor to use one or more major constituent peaks in the assessment.
  - The contractor shall evaluate the suitability of the analytical system used for the analysis relative to precision, theoretical plates, resolution, and tailing factor.

    - The analysis will use three replicates; additional replicates may be run at the discretion of the COR.
- The contractor shall calculate an RRF for each replicate analysis of the test article and the frozen reference sample of the same lot, according to the amount of test article used.
    - A mean and 95% CI shall be calculated for the frozen reference sample RRF.
    - The RRF for the test article is compared to the RRF for the frozen reference.
    - When the RRF for the test article lies outside the 95% CI of the frozen reference standard, the contractor shall inform the COR immediately.
    - When multiple chemical reanalyses have been performed on the same lot of a test article, the contractor shall plot the results, including the acceptability criteria for the analyses, as ± bounds in the graph.

At the direction of the COR, the contractor shall perform the following additional analyses on complex chemical mixtures:

- The contractor shall obtain a chemical profile of the test article using a chromatography technique coupled with a universal detector (e.g., liquid chromatography [LC] coupled with charged aerosol detection) and compare the profile to that of a frozen reference standard or an SRM as appropriate.
- The contractor shall quantitate selected marker constituents in the test article and compare them against the data generated in the CCA.

6.4. Formulation

6.4.1. Formulation Functional Area

General Requirements

For formulation activities, the following vehicles are defaults for each route used in DTT studies. The COR may propose alternate vehicles. The contractor shall not use expired feed unless approved by the COR.

- Drinking water: tap water
- Gavage:
    - Solutions: deionized water, corn oil
        - Corn oil used for animal studies shall be United States Pharmacopeia Food Grade or equivalent.
    - Suspensions: 0.5% methylcellulose in deionized water, corn oil, Cremophor EL
- Dermal: 95% ethanol, acetone
- Intravenous (IV): phosphate-buffered saline, Cremophor EL:ethanol: water (1:1:8)
- Feed: irradiated NTP-2000 meal, NIH-07 meal, Lab Diet 5K96 meal

The following containers are recommended for each dose formulation. In some instances, it may be necessary to employ alternate dose formulation containers if an analytical interference could

be introduced into a dosed animal. In these instances, approval must be obtained from the COR. The COR may request that the contractor evaluate containers for specified contaminants prior to use.

- Drinking water: plastic, polyethylene, polypropylene[7]
- Gavage: amber glass with Teflon®-lined caps
- Dermal: amber glass with Teflon-lined caps
- Feed: plastic bags

When similar vehicles are to be evaluated (e.g., NIH-07 and NTP-2000), the contractor shall perform one functional activity for both vehicles. In this situation, the contractor, in consultation with the COR, shall determine a primary vehicle.

Calculations

The contractor shall use curve-fitting techniques employing a linear regression model, with or without weighting, to determine the best fit line for the vehicle and solvent standards from the full validation and/or cross-validation study. Under certain circumstances, it may be possible to demonstrate that the analytical system is reproducibly nonlinear. In these cases, with the approval of the COR, the contractor may use a nonlinear regression model. Once the model has been chosen, the contractor shall perform the following calculations to establish the best fit line:

- Calculate the regression equations for the vehicle or solvent standard curves. Do not correct the response for experimental vehicle or solvent blank values. For spectrophotometric (i.e., ultraviolet/visible spectroscopy [UV/VIS]) determinations, zero the instrument using the solvent only and then compare the blank value with the Y-intercept obtained from the regression equation.
- Calculate the correlation coefficient r for the solvent and vehicle calibration curve. With the approval of the COR, the coefficient of determination r^2 may be calculated when it is a better measure of goodness of fit.

The contractor shall use the responses from the solvent standards, vehicle standards, and formulations, as appropriate, to calculate the precision, recovery, relative error, or measurement limits of the method as follows:

- Precision (percent relative standard deviation, %RSD):

$$\%RSD = S_{(n-1)} \div Yv_{Bar} \times 100 \quad [1]$$

  where $s_{(n-1)}$ is the standard deviation and Yv_{Bar} is the average of each vehicle standard or formulation analyzed in triplicate

- Percent recovery at each concentration according to the following equation:

$$\%Recovery = [(Yv_{ij} - Yv_{Bar\text{-}blank}) \div (Ys_{ij} - Ys_{Bar\text{-}blank})] \times 100 \quad [2]$$

[7]Drinking-water container requirements refer to storage containers, not animal drinking-water bottles. For requirements for animal drinking-water bottles, refer to the Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences.

where Yv_{ij} is the response for each of the vehicle standards, Ys_{ij} is the response for each of the corresponding solvent standards, $Yv_{Bar\text{-}blank}$ is the mean response for the vehicle blank, and $Ys_{Bar\text{-}blank}$ is the response for the solvent blank. Do not calculate recovery for blanks.

- Determine the percent relative error (RE) of each determined (calculated) concentration compared with each theoretical or target (for formulations) or nominal (vehicle or solvent standard) concentration as follows:
  - Calculate the concentration from the measured responses for the vehicle standards using the regression equation.
  - Calculate the RE using the following equation:

$$\%RE = [(Xv^{d}_{ij} - Xv^{t}_{ij}) \div Xv^{t}_{ij}] \times 100 \quad [3]$$

    where Xv^{t}_{ij} is the theoretical or nominal concentration and Xv^{d}_{ij} is the determined concentration calculated from the regression equation.

  - For vehicle standards prepared and analyzed in triplicate, compute the RE for the mean response at each concentration in addition to that for the individual standards.
- Determine the measurement limits as defined below:
  - Experimental limit of quantitation (ELOQ) shall be defined as the concentration of the lowest standard that meets the acceptability criteria of the analytical method (normally ±10% RE, with an RSD of ≤10%).
  - Limit of quantitation (LOQ) shall be defined as 10 times the standard deviation of the vehicle blank or lowest standard (ELOQ), expressed as concentration.
  - Limit of detection (LOD) shall be defined as three times the standard deviation of the lowest standard (ELOQ), expressed as concentration.

Acceptance Criteria

The following acceptance criteria must be met for analytical method validation or formulation analysis, as applicable. Deviations from the typical acceptance values listed below require the prior approval of the COR.

Method Validation

Linearity or Conformance to the Model

- Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit.
- The correlation coefficient r shall be ≥0.99 for linear fits.
  - When the coefficient of determination r^2 is an appropriate measure of goodness of fit, it must be ≥0.98.

- To achieve an acceptable fit, a data point may be discarded only after it has been shown to be statistically valid to do so. Normally this involves a Dixon's Q test[8] of all the replicates at that concentration.

Accuracy (Relative Error)

- RE shall be ≤ ± 10% of the nominal value.
- Higher RE values may be accepted but will require the approval of the COR.

Precision (Relative Standard Deviation)

- RSD shall be ≤ ± 5% of the mean found value.
- Higher RSD values may be accepted but require the approval of the COR.

Recovery

- The method will be considered acceptable when recovery at each vehicle-standard concentration is within ±20% of the corresponding solvent standard and method limits of quantitation are acceptable to quantitate the lowest anticipated formulation concentration. All vehicle standard concentrations shall have similar recoveries.
  - When the above conditions are not met at any vehicle-standard concentration, the COR must be consulted for approval.
- When there are no observed differences in response between the solvent and corresponding vehicle standard (e.g., 100% recovery), the contractor shall use a solvent calibration curve for quantitation of formulation samples per COR approval.

Measurement Limits

- Acceptability criteria for measurement limits are dependent on the study and will be provided by the COR on a case-by-case basis.

Homogeneity

A formulation shall be considered homogeneous if the RSD of samples taken from an individual blender port (top-right, top-left, and bottom) or sampling locations is < ± 5% and for the nine samples taken together does not exceed ± 5%.

The found formulation concentration of all samples must be within 10% of the target concentration.

Stability

A formulation shall be considered stable at a particular time point if the found concentration is not statistically different from the day 0 determined concentration.

When the deviation of the found concentration exceeds the statistical limit, the formulation may be considered stable if the found value is within 10% of the day 0 found concentration.

When the found concentration on day 0 differs from the target value by > ± 10%, the COR shall be immediately notified.

[8] http://en.wikipedia.org/wiki/Dixon%27s_Q_test

Formulation Analysis

Linearity

Calculate the regression equation and the correlation coefficient. Compare this analysis with all previous analyses and demonstrate that the method is reproducible. The correlation coefficient r must be ≥0.99.

To achieve an acceptable fit, a data point may be discarded only after it has been shown to be statistically valid to do so. Normally this involves a Dixon's Q test[8] of all the replicates at that concentration.

Evaluate the suitability of the analytical system used for the analysis relative to precision, theoretical plates, resolution, and tailing factor.

Accuracy (Relative Error)

RE shall be ≤ ± 10% of the target concentration.

Results that deviate from the theoretical concentration by >10% will be considered out of tolerance. There may be cases in which a 10% tolerance limit cannot be attained; these will be addressed on an individual basis and must be approved in advance by the COR. If the dose formulation is found to be out of tolerance, the COR shall be immediately notified.

Precision (Relative Standard Deviation)

The %RSD of the triplicate formulation analyses shall be ≤ 5%. If the %RSD is >5% and an outlier is suspected, Dixon's Q test shall be run. If $Q_{calc} > Q_{90}$, the outlier can be rejected. If the suspect value cannot be rejected, the contractor shall analyze a fourth aliquot and report the results of all four aliquots to the COR.

Higher RSD values may be accepted but require the approval of the COR.

6.4.2. Preliminary Formulation Studies (PFS)

At the direction of the COR, the contractor shall perform one or more of the following activities to assess the solubility, suspendability, and syringeability of a test article in vehicles:

General Requirements

The maximum concentration at which solubility or suspendability will be assessed is assumed to be 600 mg/mL. However, in most cases, the COR will specify the highest concentration to be tested, which may differ from 600 mg/mL.

If the test article is not soluble at the specified maximum concentration, and as long as it continues to be insoluble, incremental dilution with additional vehicle will be carried out to a final concentration of 0.50 mg/mL. If solubility cannot be achieved at a concentration of 0.50 mg/mL, suspendability will be assessed over the same concentration range. The final volume of the mixed test formulation shall be at least 20 mL.

Up to two aliquots of the test article will be used for all solubility and suspendability testing. One aliquot shall be used for liquid:liquid solutions; two aliquots shall be used for solid:liquid solutions.

- Aliquot 1 shall be mixed using vortex mixing. Times up to 1 minute may be used with multiple (repeat) mixing intervals.
- Aliquot 2 shall be mixed by sonication for 5 minutes. Times up to 30 minutes may be used with multiple (repeat) mixing intervals. Heating of the samples must be avoided.

When the stability of the chemical or test article is known, formulations may be warmed to a maximum of 60°C to improve the solubility of the constituents. Warmed formulations must be allowed to cool to room temperature prior to the visual assessment of solubility.

All aliquots will be carried through the Solubility Evaluation described below.

If the test article is known to have limited solubility in the vehicle, the solubility determination may be omitted and the procedure started with the syringeability determination (see below).

Solubility and suspendability can be assessed at the same time.

All references to suspensions also apply to liquid:liquid emulsions.

Solubility Evaluation

Visually estimate the maximum solubility of the test chemical in one or more vehicles, specified by the COR.

Evaluate solubility at room temperature over the concentration range prepared.

Observe and record the physical characteristics of the solution. Note specifically the consistency of the mixture and whether it remains a solution upon standing.

Store the solution for 24 hours under refrigeration and note any physical changes. If precipitation occurs after storage, determine whether warming to room temperature and mixing can achieve a usable solution.

If the test article is soluble at the maximum concentration tested, the contractor shall evaluate the syringeability of the solution (see Syringeability Evaluation).

If dissolution takes place after the addition of more vehicle or at a concentration lower than the maximum concentration tested, syringeability (see Syringeability Evaluation) shall be evaluated at the concentration nearest saturation.

Suspendability Evaluation

When the test article has been shown to be insoluble, estimate the maximum suspendability of the test chemical in one or more vehicles, at an initial maximum concentration, specified by the COR (see Section 6.4.2. General).

Observe and record the physical characteristics of the mixtures. Note specifically the consistency of the mixture and the “rate of settling” when the stirring is stopped.

Suspensions shall initially be mixed manually. Once mixed, suspensions shall be stirred with a magnetic stir bar to maintain the suspension.

Evaluate the resulting mixture, classify it according to the following criteria, and proceed as directed for each suspension class.

- Paste: Very viscous. Difficult or impossible to stir.
- Thick: Stirrable but the chemical is not easily dispersed in vehicle.
- Dispersed: Test chemical is easily dispersed in vehicle, giving an essentially uniform suspension with stirring.

Add more vehicle in small increments until the mixture can be classified as "Dispersed" or until the test article completely dissolves.

When an acceptable suspension cannot be achieved with stirring, the contractor shall prepare another at the same starting concentration, sonicate or homogenize using a polytron for 5 minutes, and evaluate the mixture to determine whether sonication changed the nature of the suspension.

If a mixture that can be classified as "Dispersed" is achieved, determine the maximum concentration that can be dispensed through a syringe fitted with a gavage needle (see Syringeability Evaluation).

If a mixture that can be classified as "Dispersed" is not achievable, the contractor shall add more vehicle in small increments to the original mixture until a "Dispersed" suspension is achieved, the test article is completely dissolved, or the lower solubility limit (0.5 mg/mL) is reached.

If the test article was determined to be suspendable and syringeable (see Syringeability Evaluation), store the suspension for 24 hours under refrigeration and note any physical changes.

If settling occurs after refrigeration, determine whether warming to room temperature and mixing can achieve a usable suspension.

Visually estimate the stability of the dose formulations for 16 days. The COR may direct the contractor to use a previously developed analytical method to evaluate stability.

Syringeability Evaluation

Syringeability shall be tested only for solutions and "Dispersed" suspensions unless directed by the COR.

Syringeability is defined as the maximum gavage formulation concentration that can be dispensed through a syringe fitted with a gavage needle when 1–3 mL of the suspension can be drawn and discharged through a gavage needle/syringe assembly while meeting the following specifications:

- Time to deliver 1 mL of a formulation shall be ≤4 seconds.
- Formulations must be dispensed within the required time to deliver without undue pressure or resistance.
- Formulations must be dispensed without noticeable differences in consistency (i.e., test article content) between the original mixture and the withdrawn aliquot, when dispensed within the required time to deliver.

Syringeability shall be evaluated using 22-gauge gavage needles. If a suspension is not syringeable through a 22-gauge gavage needle, the contractor shall evaluate 20- and 18-gauge gavage needles until the solution passes.

If the mixture is not syringeable through an 18-gauge gavage needle, dilute the suspension with vehicle, stir with a magnetic stir bar, and repeat the syringeability determination with an 18-gauge gavage needle, using successively lower concentrations until syringeability is achieved.

Using the formulation concentration found to be syringeable through an 18-gauge gavage needle, repeat the syringeability determination using 20-, 22-, and 24-gauge gavage needles and record the highest concentration that will successfully pass through all needle sizes tested.

6.4.3. Formulation Development (FD)

General Requirements

An FD plan shall be posted to the DTT PMS and approved by the COR before work described in this functional area can commence.

Preliminary indications of inhomogeneity and/or instability shall be reported to the COR as soon as they are found. The contractor shall obtain COR approval to proceed with homogeneity or stability evaluations prior to commencing work on these evaluations.

FD involves three separate but interrelated activities:

Method Development, Qualification, and/or Validation

Develop and qualify or validate an analytical method for the analysis of dose formulations for test article concentration, as directed by the COR, taking into account the expected concentration(s) of test article in the vehicle.

Homogeneity Evaluation

Determine the proper procedure for mixing the test article and vehicle to achieve homogeneous mixtures without introducing artifacts or other contaminants, evaluate homogeneity, and provide specific mixing instructions.

Stability Evaluation

Determine the stability of the test article mixed with the dosing vehicle at concentration(s) specified by the COR using the qualified or validated analytical method under conditions of long-term storage (see Storage Stability) and simulated dosing (see Dose Simulation).

Analysis Requirements

At the direction of the COR, the contractor shall perform all or some of the following activities.

Method Development and Qualification

The contractor shall develop and qualify a method for the analysis of a test article over a concentration range in a vehicle designated by the COR. The contractor shall include an IS appropriate for the analyte and the analytical method. Typically, dose concentration ranges vary from 1 to 500 mg/mL for liquid formulations and from 1 to 30,000 ppm for feed, with dilution into the analytical range at higher concentrations.

The COR will determine the acceptance of the method after reviewing the data.

For feed formulation, it must be demonstrated that the presence of rodent urine and feces in the dose-feed at a w/w concentration of approximately 5% each does not interfere with the analytical method.

Method Validation

General Requirements

When specified by the COR as part of an FD assignment, a method validation shall be conducted. These assessments will be conducted over the expected concentration range of the samples to be analyzed, which is typically a concentration equal to 90% the proposed lowest formulation concentration and up to at least 110% of the highest proposed formulation concentration for the proposed study that results in an acceptable %RSD value.

When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diets) are to be used, the contractor shall conduct a full validation for one type (e.g., NIH-07) and then conduct a partial validation in the other (e.g., NTP-2000) (see Partial Validation).

The validation shall be performed such that it provides the following information:

- An indication of the precision (i.e., %RSD) of the method at specified concentrations.
- Confirmation by statistical and/or visual inspection that the response versus concentration function is linear, or deterministically nonlinear, over the specified concentration range.
- An indication of blank vehicle contribution to responses seen in spiked vehicle determinations.
- An estimate of recovery (percent) of the test article from vehicle at specified concentrations relative to corresponding solvent standards.
- A determination of the accuracy (i.e., %RE).
- Estimates of the measurement limits (i.e., experimental limit of quantitation [ELOQ], LOQ, and LOD).
- A demonstration of the specificity of the analytical method.

The vehicle standard curve shall contain a minimum of six points, plus a vehicle control.

The contractor shall use a commercially available statistical package to perform the required statistical analysis of the data (see Section 6.4.1. General Requirements).

The contractor shall perform calculations as described under Section 6.4.1. General Requirements and meet the acceptance criteria unless otherwise specified by the COR.

Full Validation

Solvent Standards Preparation

- Prepare a series of solvent standards singly with the same final analytical concentrations as those of the vehicle standards, using two independently prepared

stock standards of different concentrations (Stock A and Stock B). The concentrations of the solvent standards shall be arranged so each standard comes from an alternate stock solution.

- Prepare a reagent blank in the same solvent as the solvent standards.
- Measure the solvent standard responses from a single analysis.

Vehicle Standards Preparation

- Prepare vehicle standards in triplicate at six different concentrations using two independently prepared stock standards of different concentrations (Stock A and Stock B). Use the same Stock A and Stock B standards prepared for the solvent standard curve. The concentrations of the vehicle standards shall be arranged so each standard comes from an alternate stock solution.
- Prepare a vehicle blank in triplicate.
- Measure the response from a single analysis using the same analytical system used for the solvent standards.

Method Verification

- If a vehicle calibration curve is used for quantitation, when the upper limit of the validated method concentration is <150% of the highest formulation concentration anticipated for the toxicology study, perform a method verification procedure as follows:
  - Prepare three replicate vehicle standards at a concentration corresponding to the highest required formulation concentration.
  - Dilute the standards with blank vehicle as appropriate such that the final concentration lies within the validated analytical method concentration range.
  - Analyze the diluted method verification standards using the validated analytical method.
- Calculate the mean formulation concentration, the %RSD, and the percent of the found concentration compared to the nominal concentration. Method verification standards must meet the same criteria as those established for validation vehicle and solvent standards.

Partial Validation

A full validation is run on the primary vehicle first. Once the method passes validation for the primary vehicle, a partial validation is run on the alternate vehicle(s). A calibration curve prepared in the primary vehicle shall be used to quantitate the samples prepared in the secondary vehicle.

The same standard stock solutions (Stock A or Stock B) prepared for the full validation shall be used if stability of stock solution has been demonstrated. If not, a fresh stock shall be prepared for partial validation.

Partial Validation Vehicle Standards Preparation

- Prepare three vehicle standards at concentrations bracketing the proposed analytical range (typically >1 order of magnitude) from a single stock solution and matching the range of the primary vehicle standards.
  - Prepare six replicate standards at a concentration corresponding to the lower limit of quantitation (LLOQ) for the validated method.
  - Prepare three replicate standards at each of the two higher concentrations.
- Prepare a vehicle blank in triplicate with and without IS.

Vehicle standard preparation methods are typically based on those used for the primary vehicle but may deviate as needed to optimize extraction efficiency and method performance.

Measure the vehicle standard response using the same analytical method as the one that was validated for the primary vehicle.

Partial Validation Solvent Standards Preparation

- Prepare solvent standards with the same final analytical concentrations as the partial validation vehicle standards.
  - Single replicates of each solvent standard shall be prepared from one stock solution.
  - Prepare a reagent blank in the same solvent as the solvent standards.
- Measure the solvent standard responses using the same analytical system used for the partial validation vehicle standards.

Partial Validation Method Verification

- If a vehicle calibration curve is used for quantitation, when the upper limit of the validated dose formulation concentration range is less than 150% of the highest dose concentration anticipated for the toxicology study, perform a method verification as given under full validation (see Method Verification).

See Section 6.4.1. General Requirements for required validation calculations and acceptance criteria for each calculated parameter.

Formulation Development and Homogeneity

Formulation Development

The contractor shall develop a method to uniformly formulate the test article in a designated vehicle.

For liquid vehicles, the minimum batch size shall be 1.0 L. The contractor may propose an alternate batch size subject to approval by the COR. For feed vehicles a V-shell blender shall be used with a minimum batch size of 20 kg. The contractor may propose an alternate batch size subject to approval by the COR.

The contractor shall avoid the use of serial dilution when preparing dose formulations unless prior approval is obtained from the COR.

Homogeneity Evaluation

A dose formulation homogeneity evaluation is required for suspensions and all dosed-feed formulations.

The analysis shall be done using the qualified or validated analytical method; however, calibration for the analysis of homogeneity samples may be performed using a minimum of three vehicle standard concentrations that bracket the nominal concentration of the sample (typically −½X, X, and +½X, where X is the nominal concentration of the homogeneity formulation sample).

When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diets) are to be used, the contractor shall conduct formulation homogeneity studies in both vehicles simultaneously whenever possible to reduce the time to complete the work and to reduce costs.

Homogeneity studies for feed formulations shall be conducted in a V-shell blender. It is acceptable to use alternate mixing procedures for premix preparations.

The evaluation shall be conducted at the lowest dose formulation concentration that is a suspension and at the highest anticipated dose formulation concentration from the supported study(ies) according to the following guidelines:

- For feed formulations, the contractor shall remove three samples for analysis from each of the top-right, top-left, and bottom V-shell blender ports.
- For suspensions, the contractor shall remove three samples from each of the top, middle, and bottom locations within the vessel used to prepare the formulations while it is being continuously stirred.
- Samples removed from each port or sampling location shall be analyzed once; replicate analyses are not required.
- The contractor shall calculate the mean, standard deviation, and RSD for the three samples from each port or sampling location and for the nine samples together from all the ports and sampling locations.

Homogeneity results exceeding the acceptability criteria (Section 6.4.1. General Requirements) must be approved by the COR prior to commencement of work on other aspects of the dose FD assignment.

Stability Evaluation

Stability studies shall mimic procedures used by the toxicology testing laboratories and supplies used shall be similar to supplies available at these laboratories (NIH 2023).

When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diets) are to be used, the contractor shall conduct stability studies in both vehicles simultaneously whenever possible to reduce the time to complete the work and to reduce costs.

Stability shall be evaluated using the qualified or validated analytical method, as appropriate; formulations prepared for homogeneity assessment can be used.

Stability shall be determined at a concentration of 10% lower than the lowest proposed concentration for a study. The COR may specify that additional or alternate concentrations be tested.

Calibration for the analysis of stability samples may be performed using a minimum of three independent standards that bracket the nominal concentration of the sample (typically −½X, X, and +½X, where X is the nominal concentration of the formulation stability sample).

Storage Stability

Formulation stability shall be measured up to 63 days, or as directed by the COR, and on days 0, 7, 14, 21, 35, 42, and 63 ± 1 day for all vehicles. Samples shall be stored sealed and protected from light at −20°C, 5°C ± 2°C, and ambient temperature. Aqueous formulations shall not be frozen in performance of stability studies unless directed by the COR. An alternate stability schedule may be used, subject to approval by the COR.

Dose Simulation

Dose simulation studies are designed to test the stability of the chemical or test article under conditions that approximate the physical, environmental, and handling aspects of dosing.

Containers used for these studies shall be similar to those in use at the study laboratories. Individual requirements for each vehicle are given below.

- Feed: Duration 7 days. Use stainless-steel powdered diet feeders with followers. Analyze on days 0, 1, 4, and 7 in the presence and absence of 5% urine and feces (typically rat).
- Gavage: Duration 3 hours. Use a single screw-cap bottle. Remove three 1 mL replicate samples every 15 minutes for 3 hours. At 3 hours, remove three replicate 1 mL samples from the formulation remaining in the container.
- Dermal: “Dosing Bottle” and “Thin Film” stability studies are required.
    - Dosing Bottle: Samples from each bottle shall be collected as described for gavage dose simulation studies under two storage regimens: (1) left open to air and (2) covered and periodically opened. The residual formulation in the dosing bottles shall be sampled and analyzed without replenishing the vehicle.
    - Thin Film: Apply a quantitative volume of the dose formulation to three glass Petri dishes. Wait 1, 3, and 5 hours and analyze the amount of test article remaining after quantitatively transferring the residue to volumetric containers.
- Drinking water: Duration 7 days. All dosed-water animal room stability studies shall be conducted in colorless glass drinking-water bottles unless otherwise specified by the COR, with Teflon-lined screw caps and stainless-steel sipper tubes. Analyze on days 0, 1, 4, and 7 or 8.

6.4.4. Formulation Analysis (FA)

General Requirements

At the direction of the COR, the contractor shall perform analyses of submitted dose formulation samples using previously qualified or validated methods and following the requirements in the Analysis Requirements section.

When samples are to be received from a DTT laboratory or researcher, the COR will provide the sample submission schedule to the contractor when it is available.

Analysis Requirements

The contractor shall analyze formulation samples within 1 week of the receipt of the samples.

If dose formulation concentrations are higher than the range that has been previously qualified or validated, the formulations shall be diluted into the validated analytical concentration range for analysis.

If dose formulation concentrations are lower than the range that has been validated, the method shall be revalidated or qualified, following partial validation procedures, over the new concentration range before the formulations are analyzed.

Analysis of dose formulations shall be conducted using the following procedures:

- Prepare two standard stock solutions of the test article from independently weighed samples. Use these solutions to prepare six single vehicle standards in duplicate at concentrations bracketing the concentration range expected for the samples such that alternate standards are prepared from each stock solution.
- If samples are to be analyzed from a single dose concentration, a single stock solution shall be prepared. Use this solution to prepare three vehicle standards at concentrations bracketing the concentration of the sample.
- Analyze the vehicle standards and a vehicle blank, if applicable.
- Analyze dose formulation samples in triplicate.
- Use the regression equation to compute the concentration of the formulations.
- When a formulation analysis results in values outside the acceptance criteria, the contractor shall document the issue in the DTT PMS and immediately notify the COR.

Referee Analysis

Occasionally it may be necessary to evaluate interlaboratory variability of a particular analytical method. In such cases, the contractor shall analyze a sample set supplied at the direction of DTT, using a validated method.

6.4.5. Formulation Preparation, Analysis, and Shipment (FPAS)

At the direction of the COR, the contractor shall perform one or more of the following: formulation preparation, formulation analysis, and/or formulation shipment, as specified below.

Formulation Preparation

At the direction of the COR, the contractor shall prepare control (vehicle) and test article dose formulations in vehicles specified by the COR using previously developed methods.

If no mixing protocol exists, the contractor shall develop a mixing procedure and submit it to the COR for approval prior to preparing the formulation.

The contractor shall label all prepared formulations with information that clearly differentiates the species and strain for which the dose is intended and each dose concentration and that differentiates dosed vehicle from control. Labels shall employ color codes and shapes in addition to printed information to aid in identifying each dose.

The contractor shall remove two minimum 35 mL aliquots (50 g for feed formulations) from each formulation prepared. One aliquot shall be stored frozen or refrigerated (based on minimum storage temperature used in the stability evaluation), and the other shall be designated for analysis.

Formulation Analysis

The contractor shall analyze all dose formulations within 2 days of the preparation or as directed by the COR. If animal room samples are anticipated, the contractor shall analyze them within 1 week of receipt but shall not exceed the period of use, identified based on stability data, for the original formulations.

Analysis of dose formulations shall be conducted following the requirements given in Section 6.4.4. Formulation Analysis (FA).

The contractor shall report dose formulation analysis data as soon as the data are available. When a formulation analysis results in values outside the acceptance criteria, the contractor shall document the issue and immediately notify the COR. The COR may direct the contractor to reprepare formulations that do not meet the acceptance criteria. When a formulation is reprepared, the deadlines for analysis and shipment of that formulation shall be based on its reformulation date.

Formulation Shipment

As directed by the COR, the contractor shall aliquot all formulations according to the requirements identified for a given study, package, and ship to a toxicology laboratory or investigator designated by the COR.

The contractor shall notify the recipient of its intent to ship and shall not ship until confirmation is received from the intended recipient.

Formulation information and other relevant documentation as directed by the COR, shall be provided with all formulations shipped by the contractor to any DTT laboratory.

All shipments shall conform to the requirements given in Chapter 3. Health and Safety and all applicable local, state, and federal regulations.

6.4.6. Vehicle Analysis (VA)

General Requirements

As directed by the COR, the contractor shall procure and perform analyses of dosing vehicles used in toxicity studies as described below.

The contractor shall develop an SOP based on the methodology used for each vehicle. The SOP shall be in force prior to the first use of the method. The SOP shall be submitted to the COR in accordance with Chapter 7. Reporting Requirements.

Vehicle analysis may be assigned to a subcontractor at the contractor's discretion, with the approval of the COR.

When a vehicle analysis results in values outside the acceptance criteria, the contractor shall document the issue and immediately notify the COR.

Acceptance Criteria

Corn oil: Peroxide value <3.0 meq/L.

Olive oil: Peroxide value <5.0 meq/L and free acidity ≤2 g/100 g oil.

Ethanol: Unequivocal identity as ethanol with a chromatographic purity ≥99.9%, excluding the water content. Benzene content <0.01 ppm.

Acetone: Unequivocal identity as acetone with a chromatographic purity ≥99.9%.

Methylcellulose: The IR spectrum of the test vehicle shall match a library reference IR spectrum for methylcellulose, and methoxy group content must be in the range of 26.0%–33.0%.

Feed: Refer to Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences for requirements (NIH 2023). COR may specify additional acceptance criteria. For example,

- Dioxins and related compounds: Total measured toxic equivalency (TEQ) shall be <0.5 pg TEQ/g feed. Total measured TEQ + ½ LOQ shall be <1.0 pg/g.
- Mycotoxins: If present, mycotoxins shall have individual concentrations ≤0.10 ppm. The total concentration of all mycotoxins present shall be ≤1.0 ppm.
- Nitrosamines: N-nitroso dimethylamine concentration shall be ≤10 ppb. Total (volatile) nitrosamine content shall be ≤15 ppb.

The COR may request analysis of other vehicles and set acceptance criteria accordingly.

Vehicle Specifications

Corn oil: Corn oil shall be commercially available and food grade. It must be purchased in batches no smaller than 2 gal. (7 kg) per chemical or test article for which it is the dose vehicle unless substantially less than this quantity is being used monthly. The COR must approve the purchase of lesser quantities. Corn oil shall be stored at 4°C ± 2°C, protected from light.

Olive oil: Olive oil shall be commercially available and food grade. It shall be stored at 4°C ± 2°C, under nitrogen headspace, protected from light. Olive oil may be stored at these

conditions as long as the measured peroxide and free acid values remain within ±10% of their day 0 values.

Ethanol: 95% ethanol:water or anhydrous synthetic ethanol, dried over molecular sieves.

Acetone: Acetone shall be United States Pharmacopeia-National Formulary grade (i.e., Sigma Product No. 650501, Spectrum Product No. HP402, or equivalent).

Methylcellulose: Methylcellulose shall be 4000 cPs cellulose methyl ester (i.e., Sigma Product No. M0512, Spectrum Product No. ME137, or equivalent). When methylcellulose is received, prepare aliquots for subsequent analysis and store in appropriate containers at −20°C.

Feed: Feed shall be certified, irradiated NTP-2000, NIH-07, or Lab Diet 5K96 meal for dosed-feed studies unless specified otherwise by the COR. Upon receipt, store feed in a cool, dry location, protected from direct sunlight. Feed may be refrigerated but shall not be frozen unless otherwise directed by the COR.

Analysis Requirements

Corn Oil

The contractor shall procure and analyze batches of corn oil using a method that is equivalent to the official method (Cd 8-53) of the American Oil Chemists Society (AOCS) (1972 and subsequent revisions). An alternate method may be used, but the COR must approve its use.

Each batch of corn oil shall be analyzed for peroxides upon receipt at the contractor's laboratory and at 12 ± 2-week intervals thereafter, while it is in use.

Corn oil labeled with a Prop 65[9] warning shall be analyzed for metal content using an approved Prop 65 analysis method or equivalent.

Olive Oil

The contractor shall procure and analyze batches of olive oil that will be used in the DTT Testing Program using a standard analytical procedure, derived from the official method (Cd 8-53) of the AOCS (1972 and subsequent revisions). An alternate method may be used, but the COR must approve its use.

Each batch of olive oil shall be analyzed for peroxides upon receipt at the contractor's laboratory and at 12 ± 2-week intervals thereafter, while it is in use. The following shall employ a standard analytical procedure, derived from the official method (Cd 3d-63) from the AOCS, 2009 for the determination of peroxides. An alternate method may be used with COR approval.

Each batch of olive oil shall be analyzed for free acidity, upon receipt at the contractor's laboratory and at 12 ± 2-week intervals thereafter, while it is in use. The contractor shall use a method derived from the official method (Chapter 2-91 Determination of Fatty Acids in Olive Oils by Capillary GLC) of the (AOCS) (1997 and subsequent revisions). Results shall be expressed as oleic acid.

[9] http://oehha.ca.gov/prop65.html

Ethanol

Each lot of ethanol shall be analyzed upon receipt at the contractor's laboratory and at 6-month intervals thereafter, while it is in use. Identity and purity shall be confirmed using appropriate analytical methods. The benzene content of ethanol used in this program must be confirmed by an independent analysis of the material using a method that has demonstrated benzene detection limits at or below the acceptability standard.

Acetone

Each lot of acetone shall be analyzed upon receipt at the contractor's laboratory and at 6-month intervals thereafter, while it is in use. Identity and purity shall be confirmed using appropriate analytical methods.

Feed

Typical feed used in rodent studies are irradiated, certified NIH-07 or NTP-2000 open formula diet rodents, unless a different diet is specified. Please refer to Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences for requirements (NIH 2023). COR may require the contractor to analyze for specific contaminants (e.g., dioxins, mycotoxins).

6.5. Biosample Analysis

6.5.1. General Requirements

The contractor shall develop or adapt from another source an analytical method to quantitate analyte(s) (e.g., test article and/or biotransformation products, endogenous substances) in a specified biological matrix.

The contractor shall evaluate analytical methods available from the literature or other sources, including previously developed methods, to determine the lowest feasible concentration that may be measured. Feasibility shall be defined as that concentration at which %RSD is <20% and %RE is ≤20%. Higher values for %RSD and %RE may be accepted but require approval from the COR.

The contractor shall employ an analytical method suitable for small sample sizes (typically 50 µL for biological fluids [e.g., plasma, urine] or 50 mg for tissues [e.g., brain, liver]).

The analytical method range (typically 0.1 to 500 ng/mL or ng/g for biological fluid or tissue, respectively) and LLOQ (typically 0.1 ng/mL for biological fluids or 0.1 ng/g for tissues) shall be specified by the COR.

The primary matrix used to develop the method shall be adult male Sprague Dawley rat unless otherwise specified by the COR.

The COR may direct the contractor to qualify or validate the method.

A plan shall be posted for approval by the COR prior to commencement of laboratory work.

- The plan shall describe the general analytical approach to the quantitation of analytes in a specified biological matrix, including, but not limited to, the following:

- Literature reference(s) that serve as a starting point for the method development.
- The proposed analytical technique, including the method parameters, if known (e.g., chromatographic separation).
- The acceptance criteria that shall be applied to the results.

All QC samples shall be prepared and stored in containers similar to those used for study sample storage.

SOPs pertaining to the analysis of samples from a study shall be submitted once the method development and/or validation work is completed.

Calculations

All or some of the following shall be estimated as appropriate for a given task.

The contractor shall use curve-fitting techniques employing a linear regression model with or without weighting. Under certain circumstances, it may be possible to demonstrate that the analytical system is reproducibly nonlinear. In these cases, with the approval of the COR, the contractor may use a nonlinear regression model. Once the model has been chosen, the contractor shall perform the following calculations:

- Calculate the regression equations for the matrix and solvent calibration curves. Do not correct the response for experimental blank values, unless approved by the COR.
- Calculate the correlation coefficient r for the solvent- and matrix calibration curves.

To estimate precision, calculate the mean and %RSD for each matrix standard analyzed in at least triplicate. Assess within-day and between-day precision.

Determine the percent recovery at each analyte concentration according to the following equation:

$$\%\text{Recovery} = [(Ym_{ij} - Ym_{Bar\text{-}blank}) \div (Ys_{ij} - Ys_{Bar\text{-}blank})] \times 100 \quad [4]$$

where Ym_{ij} is the response for each of the matrix standards, Ys_{ij} is the response for each of the solvent standards, $Ym_{Bar\text{-}blank}$ is the mean response for the matrix blank, and $Ys_{Bar\text{-}blank}$ is the response for the solvent blank.

Determine the accuracy (%RE) of each found (calculated) concentration compared with each nominal (prepared) concentration as follows:

- Calculate the concentration from the measured responses for the matrix standards using the regression equation.
- Calculate the RE using the following equation:

$$\%RE = [(Xm^{f}_{ij} - Xm^{n}_{ij}) \div Xm^{n}_{ij}] \times 100 \quad [5]$$

  where Xm^{n}_{ij} is the nominal concentration of each matrix standard and Xm^{f}_{ij} is the found concentration of each matrix standard calculated from the regression equation.

- For matrix standards prepared and analyzed in at least triplicate, compute the RE for the mean response in addition to that for the individual standards.

Assess within-day and between-day accuracy.

Determine the measurement limits as defined below:

- LLOQ shall be defined as the concentration of the lowest standard that meets acceptability criteria of the assay and should correspond to the low matrix standard.
- LOD shall be defined as three times the standard deviation of the LLOQ, expressed as concentration.

Acceptance Criteria

Linearity or Conformance to the Model:

- Calibration curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit.
- The correlation coefficient r shall be ≥0.99 for linear fits. When coefficient of determination r^2 is an appropriate measure of goodness of fit, it must be ≥0.98.
- To achieve an acceptable fit, a data point may be discarded only after it has been shown to be statistically valid to do so. Normally this involves a Q test[8] of all the replicates at that concentration.

Accuracy (RE): RE shall be ≤ ± 15% of the nominal value for all concentrations above the LLOQ and ≤ ± 20% of the nominal value at the LLOQ. Higher RE values may be accepted but will require the approval of the COR.

Precision (RSD): RSD shall be ≤ ± 15% of the mean value for all concentrations above the LLOQ and ≤ ± 20% of the nominal value at the LLOQ. Higher RSD values may be accepted but require the approval of the COR.

Recovery: The method shall be considered acceptable when recovery at each matrix-standard concentration or post-extracted spiked samples is >50% relative to the solvent standard curve. The range of % recovery values shall not exceed 20% between concentrations tested. When recovery is ≤50% at any matrix-standard concentration, the COR must be consulted for approval.

Selectivity: Any peak eluting at the retention time of the analyte or IS shall be <30% of the LLOQ response of the analyte or IS peak response.

Carryover: Carryover shall be <30% of the upper limit of quantitation concentration. Nonzero carryover values must be approved by the COR.

Measurement Limits: Acceptability criteria for measurement limits are dependent on the study and shall be provided by the COR on a case-by-case basis. Typically, the LLOQ is 0.1 ng/mL (or 0.1 ng/g).

QC Standards: At least 67% of the QC samples analyzed during an analytical run shall have concentration results within 15% of their nominal values (for QC samples prepared at the LLOQ, this requirement may be set to 20% with approval of the COR). At least 50% of the QC samples analyzed at each concentration level shall be within 15% of their nominal concentrations (for QC samples prepared at the LLOQ, this requirement may be set to 20% with approval of the COR).

A CI approach yielding comparable accuracy and precision in the run is an acceptable alternative to the requirements set forth above.

6.5.2. Biosample Method Development (BMD)

At the direction of the COR, the contractor shall perform a method qualification or validation.

The contractor shall develop a plan and submit it to the COR for approval prior to starting the laboratory work. The plan shall describe the work to be done and the acceptance criteria that shall be applied to the results.

The analyses must be performed such that it provides the following information: confirmation of the linearity (or deterministic nonlinearity) of the response versus concentration curve over the specified concentration range; blank matrix contribution to analyte response; variability associated with the measurement determined as precision and accuracy at specified concentrations; an estimate of recovery of the analyte from the matrix at specified concentrations; estimates of the measurement limits (i.e., LOD and LLOQ).

Method qualification requires method evaluation on one analysis day.

Method validation requires method evaluation on three analyses days.

When a qualified or a validated method exists for a similar tissue or biological fluid in the same species, the contractor may perform a partial qualification or partial validation (see Partial Validation). If the partial qualification or validation fails, a qualification or full validation must be performed, per approval of the COR.

Solvent Standard Preparation

Prepare duplicate standards, in addition to the blank, at a minimum of six concentrations in the biological sample extracting solvent for use as a solvent calibration curve. Use two independently prepared stock standards of different concentrations (Stock A and Stock B) to prepare the solvent calibration standards. Solvent calibration standards shall be prepared over a >10-fold concentration range.

Matrix-Standard Preparation

Use two independently prepared solvent stock standards of different concentrations (Stock A and Stock B) to prepare the matrix calibration standards in target matrix. The same stocks that were used in Solvent Standard Preparation can be used here.

Prepare triplicate matrix calibration standards at a minimum of six concentrations (excluding blank) in the biological matrix for use as a matrix calibration curve. The matrix calibration curve shall be prepared at the same concentrations as the solvent standards.

Prepare at least six replicate matrix standards at the expected lowest quantifiable matrix calibration standard concentration (LLOQ). If uncertainty exists with respect to the lowest matrix calibration standard concentration, six replicates may be prepared at additional concentrations, per COR direction.

Prepare six matrix blanks with IS (method blank) and six matrix blanks without IS (matrix blank).

Quality Control Sample Preparation

QC samples shall be prepared in triplicate in the target matrix at three concentrations corresponding to 150% of the LLOQ, a midrange concentration, and 80% of the highest calibration standard. QC samples may be prepared from a single stock, but the stock shall be a different from those used to prepare matrix calibration standards.

Analysis Requirements

Experiments for evaluation of calibration curve and assessment of stability of the processed samples during the anticipated analysis period are described below. Components may be run concurrently whenever possible to increase efficiency and minimize the time required for completion.

The following shall be determined:

- Linearity of the matrix calibration curve.
- Stability of the analytical system (instrument drift) must be determined by splitting the processed matrix calibration standards prepared on one day into two sets and by running one set at the beginning of the analytical run and the other set at the end.
- Intra- and/or interday accuracy (determined as %RE) and precision (determined as %RSD) shall be determined using QC samples run in triplicate at three concentrations.
- Selectivity shall be determined by evaluating the response of the matrix blanks.
- The LOD and LLOQ values shall be determined.
- The IS response in the method blanks shall be evaluated and the mean response and %RSD calculated. Evaluate the IS response in all analytical runs and calculate the within-day and between-day variability.
- Recovery of the analyte must be established by either (1) comparing extracted samples with corresponding extracts of blanks spiked with the analyte post-extraction at three QC concentrations (extraction efficiency) and/or (2) comparing extracted samples to corresponding solvent standards (recovery), as directed by the COR.
- If applicable, any matrix effects that are due to use of a particular analytical system (e.g., LC-MS) shall be assessed by comparing extracts of blanks spiked with the analyte post-extraction with corresponding solvent standards.
- Three method blanks shall be run after the highest matrix calibration standard to assess the carryover.
- The stability of analytes in processed (e.g., extracted sample) or prepared (e.g., headspace gas chromatography-mass spectrometry [GC-MS] analysis of whole blood) sample shall be determined over the period of analysis and under conditions of storage (Analysis Period Stability [APS]) to cover the following, as appropriate: storage duration equal to the longest expected analysis run time for the largest sample set anticipated to be run at one time (e.g., at ambient or on autosampler tray); after samples have been stored for a period of time equal to the longest expected storage period (e.g., refrigerated); after samples undergo three freeze-thaw cycles over a period of time equal to the longest expected storage period for samples. All APS

samples must be analyzed with a calibration curve. COR may request a limited APS assessment for method qualification.

- Method Verification: The contractor shall demonstrate that matrix samples with concentrations at least 10-fold higher than the qualified or validated range, or at a concentration specified by the COR, can be accurately quantified by diluting with matrix or solvent into the calibration range (dilution verification). The contractor shall use matrix QC standards prepared near the highest anticipated concentration outside the validated range to perform the verification. The COR may direct the contractor to perform additional assessments; for example, the feasibility of using a smaller sample size (e.g., 25 µL plasma) to bring the analyte concentration into validated range or using a larger sample size (e.g., 200 µL) to increase the sensitivity of detection and quantitation.

Additional Validation Requirements

Ruggedness/Cross Validation: When method transfer is anticipated, a second analyst, instrument, or column may be used as a check on the ruggedness of the method. All analyses performed here must be discussed in the report, and the data generated must be handled appropriately.

Partial Validation/Secondary Matrix Evaluation

The COR may direct the contractor to do additional studies to establish the applicability of a previously validated method in the primary matrix (e.g., SD rat plasma) for a secondary matrix (e.g., SD rat liver, HSD rat plasma, HSD rat liver) or to assess a change in the method (e.g., change in sample extraction procedure). When directed by the COR, the contractor shall assess some or all of the following: precision and accuracy of the method across the expected concentration range, LOD and LLOQ, recovery, and selectivity. If the partial validation fails, a full validation may be required at the direction of the COR.

The following sections describe assessing a validated method in a secondary matrix. Sample preparation is typically based on those used for the primary matrix but may deviate as needed to optimize extraction efficiency and method performance. Sample analysis will be done using the same analytical system used for the full validation in the primary matrix.

Prepare solvent standard concentrations as those used during full validation. Single replicates of each solvent standard shall be prepared and analyzed from one stock solution. The same stock solution (Stock A or Stock B) prepared for the full validation can be used.

Prepare six matrix calibration standards in triplicate in primary matrix from two independently prepared stock solutions at concentrations bracketing the proposed analytical range. The same stock solution (Stock A or Stock B) prepared for the full validation can be used if its stability has been demonstrated.

Prepare the following samples in secondary matrix:

- Prepare six replicate standards in the secondary matrix at a concentration corresponding to the LLOQ for the method validated in the primary matrix.
- Using one of the two primary stock standards, prepare three replicate QC samples in the secondary matrix at each of three concentrations based on primary matrix

concentrations: 200% of the LLOQ, 75% of the highest standard, and a concentration corresponding to the middle of the standard curve.

- Prepare three matrix blanks with IS (method blank) and one set without IS (matrix blank) in the secondary matrix. The COR may ask the contractor to replicate each of the two sets in up to three lots of blank secondary matrix from at least two sources.

Analyze samples using the instrument conditions used for full validation. Analytical parameters may be altered to optimize method performance, with COR approval.

Quantitate analyte in secondary matrix using primary matrix-standard calibration curve.

Evaluate the secondary matrix QC standards for accuracy, precision, and recovery.

Evaluate the response of the secondary matrix method blanks to determine selectivity.

Evaluate the responses of the secondary matrix LLOQ standards with method blanks to determine the LLOQ for the secondary matrix. When applicable, evaluate the responses of the secondary method blanks to determine the LOD.

Evaluate the reproducibility of the IS in the secondary matrix.

Method Verification: The COR may request the contractor to perform method verification in the secondary matrices as described for the primary matrix.

Stability Evaluation

Analyte stability in matrix shall be conducted using qualified or validated method as applicable. Stability assessment may vary when performed using qualified or validated method per COR direction.

The longer-term stability of analyte(s) in the primary or secondary matrix shall be established for up to 60 days under the conditions at which the study samples are to be stored prior to analysis by preparing QC samples. When storage of samples for periods >60 days is anticipated to cover study sample storage duration, the COR may direct the contractor to conduct an extended stability evaluation for >60 days.

Stability QC samples shall be prepared at 75% of the highest standard and 150% of the LLOQ in the validated concentration range. Sufficient stability QC samples shall be prepared and stored to cover triplicate analysis at time points that include, but are not limited to, 0, 15, 30, and 60 ± 2 days. Typical storage conditions include $\leq -70°C$, protected from light. Stability shall be evaluated against matrix QC samples prepared on day 0.

The COR may direct the contractor to evaluate freeze-thaw stability of the analyte in matrix after a minimum of three freeze-thaw cycles and after freezing for approximately 12 hours between cycles.

The matrix calibration curve for the analysis of stability QC samples shall be performed using a minimum of three matrix-standard concentrations that bracket each of the concentrations of the stability samples. The three-point matrix-standard curve shall be analyzed in duplicate at the same time as the QC stability samples with one replicate curve analyzed at the beginning and end

of an analytical sample set. The default for the matrix curves is the primary matrix unless the secondary matrix does not pass partial validation.

Stability of the analyte and IS solvent stock solutions shall be evaluated under their recommended conditions of storage, prior to their reuse. Analyte and IS solvent stock standards shall be analyzed over a time range that brackets their period of use.

6.5.3. Biological Sample Analysis (BSA)

Analysis of biological samples from a study shall conform to the SOP created after the method development work.

Samples shall be analyzed using a qualified or validated analytical method as directed by the COR.

Upon receipt, biological samples to be analyzed shall be stored at ≤ −70°C or under conditions that have demonstrated analyte stability in biological matrix unless prior approval is obtained from the COR to store samples under different conditions.

The blank sample matrix used for QC samples shall be the same tissue(s) from the same species and strain(s) as the samples to be analyzed. If a blank matrix that meets these criteria cannot be obtained, an alternate matrix may be used with prior approval of the COR.

If analyte stability has not been established previously, the contractor shall prepare stability QC samples in blank sample matrix at 75% of the highest standard concentration and 150% of the lowest standard concentration (LLOQ) in the validated analytical method range and store them with the samples.

- If the validated analytical method range is not known, stability QC samples shall be prepared at concentrations specified by the COR. Sufficient stability QC samples shall be prepared to allow for triplicate analyses on each analysis day.
- The contractor shall analyze one set of stability QC samples at each concentration on the day of their preparation to serve as a day 0 reference point.

The contractor shall prepare matrix QC samples in the same matrix as the study samples at two concentrations for each analytical run.

- The low concentration matrix QC sample shall correspond to the second-lowest matrix-standard concentration in the validated analytical method.
- The high concentration matrix QC sample shall correspond to the second-highest matrix-standard concentration in the validated analytical method.

Sample Analysis

- Single analysis of study samples shall be conducted.
- The contractor shall analyze at a minimum a six-concentration matrix-standard curve in duplicate with the samples.

- Matrix QC samples are to be processed with each batch of study samples. The total number of matrix QC samples analyzed shall be approximately 10% of the number of the study samples.
  - Matrix QC sample results shall be considered acceptable if they meet the %RE established in the method validation (i.e., when the validated method has shown an RE of 20%, the QC's run using that method shall also have an RE that does not exceed 20%).
  - If matrix QC samples fail to meet the acceptance criteria, the contractor shall immediately document the issue in the DTT PMS and notify the COR.
- If the storage stability of the analyte in matrix is not known, the contractor shall analyze one set of three QC stability standards at each concentration on each analysis day to assess the stability of the stored samples.
- If the reproducibility of the method has not been demonstrated, if directed by the COR, the contractor shall analyze 10% of the submitted samples for each dose group, in each matrix, as incurred samples. The contractor shall use the incurred sample results to estimate the sample reproducibility for the assay in each matrix using a Bland-Altman plot,[10] including limits of agreement.

6.6. Special Studies

6.6.1. Special Chemical Activity (SCA)

When a requirement necessitates a deviation from the descriptions for a functional activity but falls within the scope of work under the contract, the COR shall direct the contractor to perform a Special Chemical Activity.

The requirements and scope of work will be assigned by the COR. The contractor shall develop an analysis plan and receive COR approval prior to commencement of lab work.

Examples of work that may be performed under this functional activity include, but are not limited to:

- Studies on the rate of loss of test chemical from solvents or dosing vehicles.
- Studies of complex mixtures, including nontargeted analysis to compare similarities and differences between multiple lots of test articles.
- Identification of a metabolite of a test article in a biological matrix.
- Partition coefficient determination of an analyte between two matrices.
- Protein binding of an analyte (e.g., % analyte bound to plasma protein).
- Particle size reduction to submicron diameters.
- Forced degradation studies of a test article.
- Studies to determine enzyme activity (e.g., use of midazolam to determine CYP 3A4 activity, CYP 1A1 activity following exposure to a test article).

[10] http://en.wikipedia.org/wiki/Bland-Altman_plot

- Quantitation of analytes in in vitro assay (IVA) media, cells, or cell fractions.
- Activities that are combinations of two or more functional areas (e.g., chemical identity and purity determination and formulation development).
- Activities that are combinations of two or more functional activities of a given functional area (e.g., a formulation analysis that includes a validation to cover a lower concentration, which is a combination of parts of the FDV and FPAS assignment types).
- Activities that cover a subset of a functional activity or that fall within the scope of the functional activity but do not include all of the activities described for that functional activity (e.g., performing a CCA without an accelerated stability study component).
- Development and/or application of prediction models, such as physiologically based pharmacokinetics models to predict disposition of chemicals in animal or human models and in vitro to in vivo extrapolation of data to predict phenomena in vivo.
- Drafting of manuscripts and/or meeting abstracts, posters, and presentations for submittal to peer-reviewed journals or scientific conferences, respectively.

Determine the feasibility of generating a specified atmospheric concentration of a test article. The contractor shall characterize the test article prior to its use if directed by the COR.

The contractor shall perform all or a combination of following activities:

- The contractor shall investigate the production of a homogeneous atmosphere using a chemical specified by the COR and an appropriate generation system and chamber.
- The contractor shall collect samples and perform analyses to determine the concentration of a test article in an exposure chamber.
- The contractor shall determine the degree to which a test article tends to polymerize under conditions required to generate a vapor.
- The contractor shall determine the purity profile for the vapor phase of a test article in the chamber and the unvaporized test article.

6.6.2. Annual Water Analysis (AWA)

At least once per year, during periods when animal studies are being conducted, the contractor shall provide analyses of water used in the laboratory.

- The contractor shall demonstrate that water provided for laboratory animal use meets U.S. EPA National Primary Water Regulations.
- In addition, water components and contaminants listed in the Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences shall be determined (NIH 2023).
- The contractor shall provide analyses of water from an animal room or a composite from several animal rooms at least once per year.
- The contractor shall specify whether distilled and/or filtered water was used.

- A laboratory qualified to conduct such studies on a local, state, or interstate level shall perform the water analyses.

6.7. Animal Studies

Animal studies include, but are not limited to, toxicokinetic studies (TKS) and absorption, distribution, metabolism, and excretion (ADME) studies (ADMES). Animal studies typically involve single or multiple dosing of an unlabeled or labeled (stable or radioactive isotopes) test article (referred to as test article from here on) to rats and/or mice by one or more routes (e.g., oral gavage, feed, dermal, IV, drinking water).

TKS, typically conducted using unlabeled test articles, are designed to estimate various toxicokinetic parameters (e.g., half-life, clearance)[11] for a test article and/or a metabolite and bioavailability.

ADMES, typically conducted using a radiolabeled chemical, are designed to evaluate the ADME properties (e.g., % absorbed, % excreted) of a test article and/or metabolite.

The following sections describe requirements that apply to all animal studies performed. Specific requirements for TKS and ADMES functional activities are given in Section 6.7.2. Toxicokinetic Studies (TKS) and Section 6.7.3. Absorption, Distribution, Metabolism and, Excretion Studies (ADMES), respectively.

6.7.1. General Requirements

When the assignment is made, the COR shall either provide a study design or collaborate with the contractor to develop the design to address the research question.

The contractor shall prepare a study protocol based on the design and submit it to the COR for review and approval. The contractor shall obtain approval for the COR-reviewed study protocol from its Institutional Animal Care and Use Committee (IACUC) and notify the COR of the approval prior to ordering animals.

The contractor shall provide a milestone schedule that includes dates for some or all of the following at the direction of the COR:

- Submission of the study protocol
- Submission of the protocol for IACUC approval
- Animal order and expected animal receipt dates
- Proposed first and last day of dosing
- Submission of a draft final report

Applicable SOPs (e.g., the collection and analysis of biological samples) from the study shall be posted to the DTT PMS along with the IACUC-approved study protocol prior to study initiation and shall be included in the study record.

[11]Refer to Glossary of Terms.

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.

Preliminary results (e.g., toxicokinetic modeling data, palatability data) of the analysis shall be tabulated in accordance with interim data requirements found in Chapter 7. Reporting Requirements and posted to the DTT PMS upon completion of the analyses within a timeframe specified by the COR.

Animal Requirements

The contractor shall procure all animals used in animal studies unless provided by DTT. Animals used are typically rodents (e.g., Harlan Sprague Dawley rats (Hsd:Sprague Dawley® SD®) (HSD), B6C3F1 mice) and shall be procured by the contractor unless specified otherwise.

Animals used in DTT studies shall be humanely treated in accordance with the requirements set forth in Chapter 4. Animal Care and Use Requirements. Additional general requirements for animal study conduct can be found in the Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences. (NIH 2023)

The age of animals (e.g., adult animals, pregnant animals) used in studies can vary depending on the study design and will be specified by the COR.

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.

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, and Lab Diet 5K96 and shall be specified in the DTT study design.

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.

Animals shall be euthanized via exposure to 100% CO_2. Death shall be confirmed by a secondary method (e.g., exsanguination).

Test Article and Formulation Requirements

The test article shall be characterized per requirements in Section 6.3. Characterization as directed by the COR.

Refer to Section 6.4.1. Formulation Functional Area for preferred dose vehicles.

For TKS, dose formulations shall be prepared and analyzed using a qualified or validated analytical method, per requirements in Section 6.4.5. Formulation Preparation, Analysis, and Shipment (FPAS) unless specified otherwise by the COR.

Exposure Requirements

The following requirements are typical; alternate approaches must be approved by the COR.

Typical dosing volumes:

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

For dermal studies, approximately 24 hours prior to dosing, animals' backs and shoulders shall be carefully clipped to remove 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 (typically 1 × 1 cm for mice and 2 × 2 cm for rats) in the dorsal (e.g., interscapular) region. The dose site may be protected or unprotected from grooming and shall be specified in the study design. A foam appliance (rats) or metal capsule (mice) shall be used to cover the dermal dose site.

Groups of male and/or female rats and/or mice shall be exposed as specified in the study protocol via the designated route with either a single exposure or repeated (typically up to 14 days) exposures.

In IV, gavage, and dermal studies, exposure(s) should be initiated in the morning unless otherwise specified by the COR. In feed and drinking-water studies, the dosed vehicle shall be provided to animals ad libitum on the morning of study initiation and continue until study termination, as specified in the study protocol.

In repeated exposure studies or groups, as well as feed and drinking water studies, daily food and water consumption shall be measured unless otherwise specified by the COR.

Biological Sample Collection

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

Following appropriate anesthesia, biological samples (e.g., blood, tissues, excreta) shall be collected from up to three animals per endpoint, depending on the study type.

K_3EDTA shall be used as an anticoagulant for blood collection unless otherwise directed by the COR.

Biological samples collected during studies shall be stored in appropriate containers at ≤ −70°C unless otherwise specified in the study protocol.

6.7.2. Toxicokinetic Studies (TKS)

As directed by the COR, and per study design in the approved protocol, the contractor shall conduct TKS via appropriate exposure route(s).

Exposure via IV typically requires one dose. Exposures via other routes typically require three doses/exposure concentrations.

Typically, biological samples shall be collected for a minimum of 10 time points. Time points may differ by the dose/exposure concentration and/or the route.

For each group, the test article 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 are obtained

from a minimum of three animals per time point. Prior to administration, a sample of blood shall be collected to serve as a predose time point.

To minimize animal usage, blood may be collected from each animal at two or more time points, per the guidance in animal study conduct. At the direction of the COR, cannulated animals may be used to collect a complete time course. Tissue collection procedures shall be specified in the study protocol.

Concentrations of test article, metabolites, and/or other appropriate marker compound(s) in plasma and tissues collected from TKS shall be determined using a qualified or validated method, as specified by the COR and per requirements in biological sample analysis (BSA) (Section 6.5. Biosample Analysis).

The contractor, using noncompartmental 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 noncompartmental approach.

Formulation and biosample analysis data shall be included in the TKS report appendices as contributing scientist reports.

6.7.3. Absorption, Distribution, Metabolism and, Excretion Studies (ADMES)

As directed by the COR, and per study specifications given in the approved protocol, the contractor shall conduct ADMES via appropriate exposure route(s).

If a radiolabeled test article is used, radiochemical purity of the test article shall be determined by the contractor prior to the start and during an ADME study. The radiochemical purity shall be ≥90% unless otherwise approved by the COR.

The identity and purity of the unlabeled test article used shall be determined prior to use in studies.

Exposure via IV typically requires one dose. Exposures via other routes typically require three doses/exposure concentrations. For each group, the test article 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 are obtained from a minimum of three animals per time point.

Formulation and analysis method and formulation stability shall be investigated by the contractor prior to study start. Previously developed analytical methods and/or methods using radiolabels shall be used as appropriate for the test article being investigated.

To prepare dose formulations for an ADME study using radiolabeled test article, the radiolabeled test article shall be diluted with unlabeled test article such that the total radioactivity used shall be kept constant, not to exceed 50 μCi per rat and 10 μCi per mouse unless otherwise specified by the COR. The radiochemical concentrations in formulations shall be checked prior to and after dosing. The exact administered dose and the radioactivity shall be reported.

Animals shall be acclimatized individually in metabolism cages that allow for the separate collection of excreta for approximately 24 hours prior to dose administration. Following dose administration, animals shall be returned to metabolism cages. They shall continue to be individually housed in metabolism cages until the study is complete.

Excreta, blood, plasma, and tissues shall be collected at times specified in the study protocol; weights (or volumes) shall be recorded for each collection period (see below) and stored separately per time point.

Typical collection periods (hours) for excreta (e.g., urine, feces, exhaled volatile organic compounds [VOCs], CO_2) include urine: 0–8, 8–24, and every 24 hours thereafter; feces: 0–8, 8–24, and every 24 hours thereafter; VOCs and CO_2: 0–4, 4–8, 8–12, 12–24, and every 24 hours thereafter. Control samples of excreta shall be collected during the acclimation period to determine background radioactivity.

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 (the last rinse shall be with ethanol), and rinsates shall be collected separately from urine.

Volatile compounds shall be collected at or below 0°C 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. Based on preliminary data, if <1% of the total administered radioactivity is found to be in the traps, the collection of expired air shall terminate in subsequent studies following COR approval.

Tissues (glands and organs in their entirety) collected typically include adipose (perirenal), skeletal muscle (hind leg), skin (abdominal), liver, lung, kidneys, brain, spleen, heart, pancreas, thyroid, urinary balder, testes, uterus (with fallopian tubes), ovaries, small intestine, large intestine, stomach, cecum, and dose site skin (in dermal studies). 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.

Contents from the gastrointestinal (GI) tract (small intestine, large intestine, stomach, and cecum) shall be removed, combined and weighed, and stored separately from the GI tract tissues.

GI tissues shall be rinsed with deionized water unless directed otherwise by the COR, and the rinsate shall be combined with the contents.

The carcasses remaining after tissue collection shall be saved for possible further sampling or solubilization of whole carcass for mass balance determinations.

The contractor shall analyze all samples in triplicate for total radioactivity using liquid scintillation spectroscopy, either directly (after dissolution in a scintillation cocktail), after oxidation in a sample oxidizer, or after solubilization. If an unlabeled test article was used, the contractor shall analyze samples singly using a qualified or validated analytical method for specified analytes (e.g., test article, metabolite).

The contractor shall perform metabolic profiling (e.g., radio profiling, MS profiling) before and after enzyme (e.g., glucuronidase, sulfatase, acylase) deconjugation and metabolite identification in urine or other matrices as specified in the study protocol and directed by the COR.

Metabolite identification shall be done using MS, NMR spectroscopy, or other appropriate analytical techniques in urine and/or other matrices as directed by the COR.

6.8. Omics

As directed by the COR, the contractor shall perform omics analyses (e.g., exposomics, metabolomics, proteomics, lipidomics) in a variety of sample types (e.g., biological matrices, environmental samples) using appropriate analytical systems (e.g., LC coupled with high-resolution mass spectrometry [HRMS]).

When any omics assignment is performed, the requirements and scope of work will be assigned by the COR and described in the DTT PMS.

The contractor may develop appropriate methods and approaches or use those published in the literature as applicable.

The COR may direct the contractor to perform one or more activities in a single assignment.

Raw data from analysis (e.g., NTA) shall be submitted to the COR in an appropriate file format (e.g., mzML).

A plan, including the milestone schedule, shall be developed and posted to the DTT PMS for approval by the COR. The plan shall describe the general approach, sample preparation and/or assays to be used, and analyses to be performed.

Activities in this functional area include but are not limited to:

- NTA to acquire features (e.g., mass/charge) in samples and, when appropriate, to compare features between different categories of samples (e.g., plasma from animals unexposed (control) and exposed to a test article using statistical methods (e.g., principal component analysis [PCA]).
- Use NTA data to identify analytes (e.g., environmental contaminants, test article-specific metabolites, endogenous metabolites).
- Identification can be achieved using a combination of authentic standards and web-based or in-house libraries/databases.
- Identification of analytes may involve levels of confidence that conform to the five-tier guidelines (e.g., https://doi.org/10.1021/es5002105) or as specified by the COR.
- Targeted analytical approaches to quantify analytes identified in consultation with the COR.
- For quantification, analytical method(s) may be adapted from the literature, developed by the contractor, and subsequently qualified and/or validated, per direction from the COR, per requirements in Section 6.5. Biosample Analysis.
    - The COR may direct the contractor to perform validation under a BMD task.
    - The COR may direct the contractor to perform quantification under a BSA task.

6.9. In Vitro Assays (IVA)

As directed by the COR, the contractor shall perform IVAs. IVAs typically include activities related to determining the ADME and toxicokinetic properties of a test article in vitro in rodent and human models.

When any IVA assignment is performed, the requirements and scope of work will be assigned by the COR and described in the DTT PMS.

The contractor may use commercially available systems or develop appropriate systems or assays in-house.

The contractor may be required to perform one or more activities in a single assignment.

Replicate samples (n = 3) shall be used unless otherwise specified by the COR.

Analysis may require NTA approaches and targeted analysis methods. The contractor may develop methods and/or adapt methods from the literature.

For quantitation of analytes (e.g., in clearance studies), the contractor may develop and qualify methods and/or adapt methods from the literature per requirements in Section 6.5. Biosample Analysis.

The plan shall describe the general approach, assays, and analyses to be performed.

Activities under IVAs include, but are not limited to:

- Performance of assay(s) using suitable model(s) to determine absorption of a test article (e.g., Caco-2 monolayer model to determine intestinal absorption of a test article).
- Performance of assay(s) using suitable model(s) to identify transporters responsible for ADME of a test article (e.g., Pgp Glo™ assay to determine P-Glycoprotein substrates).
- Performance of assay(s) using suitable model(s) to determine clearance and/or metabolism of a test article (e.g., cryopreserved primary rodent hepatocytes to determine hepatic clearance and metabolism).
- Performance of assay(s) using suitable model(s) to determine bioaccumulation of a test article following repeated exposure (e.g., HepatoPac® or plateable hepatocyte model).

6.10. References

Roberts GK, Stout MD. 2023. Specifications for the conduct of toxicity studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences. Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Service, National Institute of Environmental Health Sciences. https://doi.org/10.22427/NIEHS-00