CCPSE Guidelines for Pharmaceutical Drug Testing on Zebrafish

Introduction

This document outlines comprehensive workflow guidelines for testing pharmaceutical drugs on zebrafish (*Danio rerio*) in compliance with Organization for Economic Co-operation and Development (OECD) testing guidelines, the Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 guidelines, and the Canadian Council on Animal Care (CCAC) Guidelines on: The Care and Use of Fish in Research, Teaching and Testing. Zebrafish have emerged as an important vertebrate model for drug discovery and toxicology research due to their genetic similarity to humans, rapid development, transparent embryos, and amenability to high-throughput screening. The regulatory framework encompasses OECD Guidelines which are internationally accepted standards for chemical testing including pharmaceuticals; ARRIVE 2.0 Guidelines which provide essential reporting standards for animal research; and CCPSE (Consortium for Coordination of Pre-Standard Research for Environmental-friendly Fish Bioassays) recommendations for fish bioassays. This integrated approach ensures scientific rigor, animal welfare, and reproducibility of results in preclinical pharmaceutical testing using zebrafish models.

Pre-study Planning

Effective pre-study planning begins with clearly defining primary and secondary objectives and formulating specific, testable hypotheses that align with the 3Rs principle (Replacement, Reduction, Refinement). Consideration should be given to whether embryonic or larval stages can be used instead of adult fish, as many jurisdictions do not consider zebrafish as protected animals until they reach the free-feeding stage (approximately 5-7 days post-fertilization). A detailed study protocol must be prepared that includes justification for animal use, study design and methodology, sample size calculation with power analysis, inclusion/exclusion criteria, definition of experimental units, randomization procedures, blinding methods, primary and secondary outcomes, and a statistical analysis plan. The protocol must be submitted to an Institutional Animal Care and Use Committee (IACUC) or equivalent ethical committee, addressing all ethical considerations and obtaining formal approval before beginning any animal work. In accordance with ARRIVE 2.0, the study should be registered in appropriate databases before initiation. All personnel involved must be properly trained and qualified in zebrafish handling and husbandry techniques, with documented training records for all researchers and technicians, and specific responsibilities assigned to team members. The developmental stage of the zebrafish to be used should be clearly specified, as different regulatory requirements may apply depending on whether embryos, larvae, or adult fish are used in the study.

Experimental Design

The experimental design should begin with selecting an appropriate study design (parallel group, dose-response, etc.), incorporating appropriate controls (vehicle, positive, negative), and defining treatment groups and duration. OECD Test Guidelines 236 (Fish Embryo Acute Toxicity Test) and 215 (Fish, Juvenile Growth Test) provide specific guidance for zebrafish studies. An a priori power analysis must be performed to determine optimal sample size, considering effect size, power

(typically 80-90%), and significance level (typically $\alpha = 0.05$). The methods and tools used for calculation should be documented, and pilot studies considered if limited data is available for power calculations. Proper randomization methods must be implemented for allocation to experimental groups, order of treatments, order of assessments, and tank arrangements. The randomization method should be documented in detail, using appropriate software or tools. Due to the large numbers of animals often used in zebrafish studies, group randomization may be applied (e.g., randomizing tanks rather than individual fish). Blinding should be implemented at multiple levels, including personnel administering treatments, personnel conducting assessments, and data analysts. Blinding procedures and verification should be documented, maintained until after data analysis when possible, and any instances where blinding is compromised should be recorded. Standardized phenotypic assessment methods should be established for evaluating effects, particularly for embryonic and larval stages where automated imaging and analysis systems may be employed.

Animal Care and Housing

Zebrafish should be sourced from reputable suppliers or institutional breeding colonies with defined genetic background, with documentation of strain, wild-type or transgenic status, age, and developmental stage. For breeding colonies, a detailed record of the genetic background and breeding history should be maintained. An adequate acclimation period should be allowed, with embryos typically collected from breeding tanks and staged according to established developmental criteria. Housing conditions must comply with OECD and CCAC guidelines, maintaining appropriate water temperature (26-28.5°C), pH (6.8-7.5), conductivity (300-1500 μS/cm), dissolved oxygen (>80% saturation), ammonia (<0.02 mg/L), nitrite (<0.1 mg/L), and nitrate (<50 mg/L). A controlled photoperiod should be maintained (typically 14 hours light:10 hours dark). Water parameters should be documented and monitored daily, with appropriate filtration systems in place. Environmental enrichment appropriate for zebrafish should be provided in adult housing systems, including plants (real or artificial), substrate, or shelters. Feeding regimes should be standardized and documented, with appropriate food types for the developmental stage (paramecia or rotifers for larvae, commercial fish food for adults). The feeding schedule and any feed restrictions should be documented, and feed consumption monitored. For larvae and embryos, appropriate embryo medium should be used (e.g., E3 medium or system water), with regular renewal to maintain water quality. Stocking density should be appropriate for the size and age of fish, with overcrowding avoided. For embryo studies, standard incubation conditions in petri dishes or multi-well plates should be established, with regular renewal of medium.

Test Compound Preparation and Administration

A chain of custody for test compounds must be maintained, documenting chemical identity (CAS number if available), physical characteristics, purity and stability, storage conditions, and expiration date. Compounds should be prepared in appropriate containment facilities. Solubility in aqueous medium should be determined, as this is critical for zebrafish exposure studies. A vehicle suitable for aquatic exposure should be selected, with DMSO being commonly used but not exceeding 0.1% final concentration to avoid toxicity. Detailed documentation of formulation procedures

should include concentrations, pH adjustments, solubilization methods, and sterilization methods (if applicable). The homogeneity and stability of the formulation in fish water should be verified, and fresh formulations prepared when stability is a concern. Dose selection should be based on previous toxicology data, pharmacokinetic data, expected clinical exposure, and required safety margins. A range-finding study is often necessary to determine appropriate dose levels. At least five concentration levels should be included for dose-response studies, with documented rationale for dose selection. The exposure method should be appropriate for the developmental stage, with static, semi-static, or flow-through systems being options for compound administration. For embryo and larval studies, compounds are typically added directly to the water in multi-well plates, while adult studies may use tank water exposure with precise documentation of the exposure system, nominal and measured concentrations, exposure duration, renewal schedule (if applicable), and monitoring of water parameters during exposure. Administration techniques should be standardized across all treatment groups, with personnel thoroughly trained in exposure methods, and any difficulties during administration recorded.

Data Collection and Observations

Observations should be conducted at standardized times, with documented frequency and timing. Observers should be trained in recognition and scoring of zebrafish phenotypes and behaviors, using standardized scoring systems. For embryonic and larval stages, key developmental endpoints should be assessed including survival, hatching rate, edema, malformations, heart rate, spontaneous movement, and touch response. For adult fish, systematic observations should be conducted for swimming behavior, appearance, feeding behavior, and any signs of distress, using validated scoring systems when available and recording observations in standardized forms. For high-throughput screening applications, automated systems for phenotype analysis and behavior tracking should be validated and calibrated. Larval locomotor activity can be measured using automated tracking systems, with parameters such as swimming distance, velocity, and movement patterns. Specialized assessments should be conducted as required, including cardiovascular function (heart rate, blood flow), neurological function (startle response, thigmotaxis), or other physiological parameters relevant to the study objectives. Image acquisition for morphological analysis should follow standardized protocols, with consistent positioning and magnification. Sample collection timing should be standardized relative to dosing and feeding schedules, with minimal handling stress. All observations should be recorded in standardized formats, with digital systems preferred for data integrity and traceability.

Endpoint Measurements

For embryo and larval studies, standard developmental toxicity endpoints should be assessed according to OECD TG 236, including coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat. Additional morphological endpoints may include body length, eye size, yolk sac size, presence of edema, spine curvature, and fin development. Behavioral endpoints may include spontaneous movement, touch response, swimming patterns, and response to light or acoustic stimuli. For later stage larvae and adult studies, growth parameters should be measured including body weight and length. For specific mechanistic studies,

additional endpoints should be included such as histopathology, gene expression analysis, or biochemical measurements. Tissue collection should follow standardized procedures appropriate for the intended analyses, with rapid processing to preserve tissue integrity. For histopathology, proper fixation (typically 4% paraformaldehyde for zebrafish) and processing techniques should be employed, with sections prepared using appropriate staining methods. Evaluations should be performed by qualified personnel, with peer review considered for critical findings. For molecular analyses such as gene expression, appropriate sample preservation (e.g., RNAlater or flash freezing), extraction methods, and analytical techniques should be employed with proper quality controls. Specialized endpoints such as behavioral analysis, cardiovascular assessment, or neurological function testing should use validated methods with appropriate controls, with all procedures documented in detail. For higher-tier studies, additional endpoints such as bioaccumulation, metabolism, or specific organ toxicity may be included, depending on the study objectives.

Humane Endpoints and Euthanasia

Specific, objective criteria for humane endpoints should be defined before study start, including parameters such as loss of equilibrium, abnormal swimming, respiratory distress, severe morphological abnormalities, lack of response to stimuli, and inability to feed. These criteria should be adapted to the developmental stage being studied, with embryonic stages primarily focusing on developmental abnormalities and later stages including behavioral and physiological parameters. All personnel should be trained to recognize these criteria, and application of humane endpoints documented. For embryonic stages prior to independent feeding, appropriate methods for cessation of development should be employed, such as fixation or rapid freezing. For post-hatch larvae and adult fish, euthanasia methods should be consistent with CCAC guidelines and AVMA Guidelines for the Euthanasia of Animals, with rapid chilling (2-4°C) followed by chemical methods (e.g., overdose of tricaine methanesulfonate, MS-222) being commonly used for zebrafish. MS-222 should be used at appropriate concentrations (typically >250 mg/L) with buffering to neutral pH using sodium bicarbonate. Personnel should be thoroughly trained in euthanasia methods, with confirmation of death documented using appropriate criteria such as cessation of gill movement, heart function, and lack of response to stimuli. The method, time, and confirmation of death should be documented. Tissue collection requirements should be considered when selecting the euthanasia method, as some methods may interfere with subsequent analyses.

Data Analysis and Reporting

A data management plan should be implemented, using validated electronic data capture systems when possible, with particular attention to the large datasets generated by high-throughput zebrafish screening. Quality control procedures for data entry should be included, with secure data storage and appropriate backups maintained. Any data corrections should be documented with reason and date. The pre-specified statistical analysis plan should be followed, accounting for any tank effects or clustering in the analysis. For high-throughput screening, appropriate correction for multiple testing should be applied. Missing data should be handled appropriately, and blinded data review considered before unblinding. All pre-specified endpoints should be analyzed using appropriate statistical methods that

account for the hierarchical structure of the data (e.g., fish nested within tanks), multiple comparisons, and repeated measures when appropriate. For dose-response studies, appropriate modeling approaches should be used (e.g., benchmark dose modeling). Any deviations from planned analyses should be documented. Reporting should follow ARRIVE 2.0 guidelines completely, including all required sections such as study design, sample size calculation, inclusion and exclusion criteria, randomization and blinding details, outcome measures, statistical methods, baseline data, numbers analyzed, outcomes and estimation, and adverse events. Special attention should be given to reporting zebrafish-specific information including strain details, husbandry conditions, water parameters, and developmental stages. All results should be reported regardless of significance, including individual or tank-level data when feasible, and all conflicts of interest disclosed. Any unexpected findings or mortality should be fully disclosed and discussed.

Quality Assurance

Detailed Standard Operating Procedures (SOPs) should be developed and followed for all procedures including zebrafish husbandry, breeding, experimental exposures, phenotypic assessment, and euthanasia. SOPs should be reviewed and updated regularly, with any deviations documented. Calibration records should be maintained for all equipment, including water quality monitoring systems, temperature controllers, automated phenotyping platforms, and analytical instruments. Computerized systems should be validated, and regular maintenance performed according to manufacturer recommendations. Regular monitoring procedures should be implemented for water quality parameters, with protocol deviations documented and a study master file maintained with all critical documentation. A system for zebrafish health monitoring should be established, with regular health checks and appropriate quarantine procedures for new fish. Comprehensive archiving procedures should be developed for raw data, study protocols and amendments, final reports, correspondence with ethical committees, test articles, and specimens as appropriate. Digital image data should be stored with appropriate metadata and in non-proprietary formats when possible. Archives should be maintained according to regulatory requirements, typically for a minimum of 5 years. For studies conducted under Good Laboratory Practice (GLP), additional quality assurance requirements apply, including independent quality assurance audits and formal certification of facilities.

References

The workflow guidelines are based on several authoritative sources, including OECD Test Guideline 236: Fish Embryo Acute Toxicity (FET) Test (2013); OECD Test Guideline 215: Fish, Juvenile Growth Test (2000); The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research by Percie du Sert N, et al. (2020), published in PLoS Biology; Canadian Council on Animal Care (CCAC) Guidelines on: The Care and Use of Fish in Research, Teaching and Testing (2005); Consortium for Coordination of Pre-Standard Research for Environmental-friendly Fish Bioassays (CCPSE) recommendations; The Principles of Humane Experimental Technique by Russell WMS and Burch RL (1959); AVMA Guidelines for the Euthanasia of Animals (Latest Edition); and Zebrafish International Resource Center (ZIRC) recommendations for zebrafish husbandry and health monitoring.