Guidelines for the Use of Non-Pharmaceutical Grade Compounds in Laboratory Animals

Investigators are expected by regulatory authorities to use pharmaceutical-grade compounds (PGC) in animals when they are available. Whenever possible, pharmaceutical grade substances must be used for compounds selected for medical treatment or to prevent or reduce animal pain or distress[2]. The use of non-pharmaceutical grade substances may be necessary to meet the scientific goals of a project or when pharmaceutical grade substances are not available. This is consistent with the regulatory requirements and the expectations of the public that research animals will be provided with *adequate veterinary care* [1-3, 8]. The Guidelines presented here are intended to help investigators understand their responsibilities and comply with federal law when the use of non-pharmaceutical-grade compounds (non-PGCs) is necessary to complete their research objectives. ***The requirements below also apply to a pharmaceutical-grade product modified by the investigator (i.e., diluted, combined, etc.):***

For all agents administered to animals, the following must be considered in the order presented for pharmaceuticals and reagents of all kinds prior to use[4]:

1. FDA approved veterinary or human pharmaceutical compounds;
2. FDA approved veterinary or human pharmaceutical compounds used to compound a needed dosage form;
3. USP/NF, BP, or other pharmacopeia recognized PGCs used in a needed dosage form;
4. Analytical grade bulk chemical [>95% pure by weight of the active chemical] (USP-NF <797> Pharmaceutical Compounding-sterile preparations) used to compound a needed dosage form (requires justification); and
5. Other grades and sources of compounds (requires justification).

Guidance for Investigators:

It is critical that sufficient information be provided in an Animal Study Proposal (ASP) for all substances administered to an animal for the IC Animal Care and Use Committee (ACUC) to effectively evaluate the safety of the agent. Provision of an optional table like the one below often ensures sufficient information for ACUC assessment of administered substances:

Item Number	Pharmaceutical or Chemical Name[a]	Source[b]	Form Obtained[c]	Pharmaceutical Grade (Y/N)[d] If "N" Provide Grade to be Used	Justification for Use of Non-Pharmaceutical Grade Compound[e]	Modifications for Use[f]	pH and Tonicity of Final Product[g]	Precautions to Ensure Sterility[h]	Dose[i]	Route, Volume, and Frequency of Administration[j]	Other Significant Considerations[k]	Reference(s) and Prior Experience
1												
2												

a) Do not provide the brand name of the agent.
b) DVR Pharmacy, Sigma Chemical, Tocris, Fisher Scientific, Millipore, etc.
c) Suspension, emulsion, dry powder, liquid, tablet, capsule, etc.
d) If you are purchasing the substance from a medical supply company, a drug wholesale supply, or directly from the manufacturer and the substance is supplied as a product intended for use in medical or veterinary patients, that substance is likely "pharmaceutical grade". In most cases, bulk reagents purchased from chemical supply companies, or the substance is designated for use in research only, that substance is most likely NOT pharmaceutical grade[4]. If you are unsure, consult your Institute Veterinarian or the DVR pharmacist.
e) Justification must address why you need to use this compound and why a pharmaceutical grade could not be used. Cost-savings alone is not adequate justification for the use of a non-PGC when

a PGC alternative exists and is available[3]. Examples of acceptable scientific justifications for the use of non-PGCs[4-6] include:

- A PGC is not available; this includes new investigational compounds.
- A PGC is not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.
- A PGC is available but does not meet the non-toxic vehicle requirements for the specified route of injection.
- The non-PGC is required to generate data that are part of an ongoing study or to generate data that are comparable to previous work.

f) Will the compound be altered in any way prior to being given to the animal (diluted, dissolved, mixed with food or flavoring, etc.)? If the compound will be diluted, mixed with another substance, suspended, dissolved, or mixed into the animal's drinking water or food, or otherwise altered, please describe what will take place, the solvent/vehicle to be used and final concentration[10,11]. If the drug will be administered directly to the animal, as supplied by the manufacturer for that purpose, then nothing needs to be specified. *Diluents, excipients, or vehicles* administered to animals in biomedical research should be pharmaceutical grade, if available[4]. Therefore, when not available they must also be handled as a non-PGC. Products administered orally, which are not pharmaceutical grade, should be food-grade[4].

g) Provide the pH and tonicity of the final product to be administered if the product will deviate from neutral pH or isotonic or does not use a buffered and/or isotonic diluent or vehicle.

h) Any compounds delivered by parenteral injections, must be sterile[9]. It is not necessary to sterilize medications that will be delivered orally or topically unless required by scientific study design. If the compound cannot be sterilized, scientific justification as to why the material cannot be sterilized and describe what techniques will be used to ensure the final product is free of unwanted pathogens, pyrogens (such as endotoxin) or contaminants that might impact animal welfare. Good aseptic technique in the preparation of all administered compounds is critical. In addition, storage conditions and expiration/use by date are also very important to protecting the integrity, purity, sterility, and stability of the compounds being administered and should be described in the ASP. There are several acceptable methods for sterilization but the two most used are autoclaving and filter sterilization. Aseptic technique is again critical as there are limits to what sterilization can remove[7].

- Autoclaving uses moist heat and pressure to sterilize components. Care must be taken when autoclaving liquids to ensure that the final concentration is appropriate and that an unacceptable amount of water has not boiled off. The compound must also be heat-stable so that it is not destroyed by autoclaving.
- Filter sterilization involves passing the final solution through a 0.22-micron pore or smaller filter into a sterile container. Examples of filters for sterilization of solutions include:
    - Positive pressure filter for filtration of large volumes (up to 2 L) Sterivex™ Filter Units - Sterile Filtration (emdmillipore.com)
    - Vacuum Filtration system for volumes up to 500 mL Stericup Quick Release-GP Sterile Vacuum Filtration System | S2GPU05RE (emdmillipore.com)
    - Syringe mounted filter for filtration of small volumes (1-100 mL) https://www.emdmillipore.com/US/en/product/Millex-Syringe-Filter-Units-Sterile-4-13-25mm,MM_NF-C9085?CatalogCategoryID=#ordering-information
    - Centrifuge filters for filtration of very small volumes (0.5 mL) https://www.emdmillipore.com/US/en/product/Ultrafree-MC-Centrifugal-

Filter,MM_NF-UFC30GV0S

i) Dose (ex: mg/kg body weight, etc.) or dose range.
j) Route of administration (ex: intraperitoneal injection, intravenous infusion, oral gavage, topically, in the drinking water, etc.), volume to be administered, frequently of administration (ex: daily, twice daily, every hour, once per week, etc.), and for how long will you give the compound (ex: one dose, for 3 days, for one week, for one month, etc.).
k) Describe any expected outcomes, possible side-effects, toxicities, etc.

Guidance for ACUCs:

Where the use of non-PGCs may be essential for the conduct of science, the goal of the IC ACUC should be to consider the health and well-being of the animals while aiding the researcher in minimizing potentially confounding experimental variables and maximizing reproducibility of the research.[2] Additional considerations include:

Consider the purpose for which a particular compound is being given:

- When compounds are used for the clinical treatment of animals or to prevent or reduce/eliminate animal pain or distress, PGCs must be used whenever possible.[1,2]
- When compounds are used to accomplish the scientific aims of the study PGCs are preferred if available and suitable. [1-4,8]

Consider the justification for any non-pharmaceutical-grade compounds to be used:

- The use of non-PGCs in laboratory animals must be described and justified in the Animal Study Proposal (ASP) and/or covered by an IC ACUC-approved policy.
- Examples of acceptable scientific justification for the use of non-PGCs include:
    - No equivalent veterinary or human drug is available for experimental use. The highest-grade equivalent chemical reagent should be used and formulated aseptically, with a non-toxic vehicle, as appropriate for the route of administration.
    - Although an equivalent veterinary or human drug is available for experimental use, the analytical or chemical grade reagent may be required to replicate methods from previous studies.
    - Although an equivalent veterinary or human drug is available, dilution, concentration or change in formulation is required.
    - If the formulation as provided must be diluted, altered by addition, or otherwise changed, there may be no additional advantage to be gained by using the USP formulation.
    - In this situation, use of the highest-grade reagent may have the advantage of single-stage formulation and result in purity that is equal to or higher than the human or veterinary drug.
    - The available human or veterinary drug does not meet the non-toxic vehicle requirements for the specified route of administration.

Additional considerations for the use of drugs/chemicals/reagents in animals:

- Whether the chemical properties of the compound are appropriate for the study and the route of administration (e.g., the purity, grade, sterility, stability in and out of solution, solution vehicle properties, pH, osmolality, pyrogenicity and compatibility of the solvent and other components of final preparation). [10,11]
- The method of preparation, labeling (preparation and use-by dates), administration and storage of formulations should be appropriately considered with the aim of maintaining their stability and

quality.

- Use must be compliant with applicable national or regional regulatory guidelines and requirements and the requirements of relevant funding agencies.
- The potential for side effects and adverse reactions should also be evaluated along with how animals will be monitored to detect these events and what, if any, treatments may be required.
- Do these considerations apply to non-survival studies? Although the potential animal welfare consequences of complications are less evident in non-survival studies, the scientific issues remain the same as in survival studies and therefore apply to non-survival studies. The use of a non-pharmaceutical-grade euthanasia agent must meet the same standards as for use in any other application.[3]
- Do these considerations apply to the vehicle/diluent/excipient as well? The guidelines pertain to all components, both active and inactive, contained in the preparation to be administered. Therefore, the vehicle used to facilitate administration of a compound is as important of a consideration as the active compound in the preparation.
- Veterinary and human drugs that are reconstituted in a manner not in accord with the product insert are considered non-PGCs.

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Approved – 12/03/2008
Revised – 12/08/2010; 04/10/13; 04/27/16, 04/28/2021, 04/26/2023

Appendix A: Tribromoethanol (Avertin) Anesthesia in Mice

Tribromoethanol is an injectable anesthetic agent once manufactured specifically for anesthetic use under the trade name Avertin. The manufactured product is no longer available. As a result, investigators who wish to use Tribromoethanol as an anesthetic must make their own solutions from non-pharmaceutical grade chemicals. This fact, coupled with the agent's potential for side effects and the abundance of alternative anesthetics currently available, has led to the recommendation that Tribromoethanol not be routinely used for rodent anesthesia. **If used must be justified within an ASP and approved by the IC ACUC.**

Tribromoethanol is not generally recommended for rodent anesthesia for the following reasons:

a. Tribromoethanol is no longer available as a pharmaceutical grade substance. According to ARAC and OLAW guidelines, investigators are expected to use pharmaceutical grade substances when possible.
b. There are well documented potential side effects of using Tribromoethanol as an anesthetic including:
    i. Variability of anesthetic effectiveness dependent upon the procedure being conducted
    ii. Potential for peritonitis and ileus
    iii. Potential for nephrotoxicity and hepatotoxicity
    iv. The breakdown products are irritating to tissues and can cause abdominal adhesions, peritonitis, ileus, and death.[1]
    v. Unknown mechanism of action
c. Ketamine combinations are safer alternatives that provide secure and stable anesthesia along with an enhanced analgesic component, which tribromoethanol does not provide.

If used for survival or non-survival procedures, use of Tribromoethanol must be justified in the ASP and approved by the ACUC. This justification should include an explanation of why other, more appropriate anesthetics cannot be used. The ASP must reference this document or describe the deviation for the preparation, storage, and use of tribromoethanol solution. It is preferred that a working solution of tribromoethanol is prepared fresh for each use, but storage for up to 1 month is permitted given appropriate storage conditions. Preparation must consider steps to maintain sterility of the final product for injection. Recommendations for mixing and storing are provided below; if these tips are followed they should minimize adverse consequences, when Tribromoethanol is used for brief surgical procedures.

Use of Tribromoethanol for more than one procedure in the same animal may lead to a higher incidence of side effects. Repeated injections of Tribromoethanol, greatly increase the risk of significant peritonitis, ileus, and death.

Mixing, Storage and Dosage Recommendations

Only properly trained and qualified individuals should prepare drugs or perform anesthesia. When diluting and mixing chemical compounds, as opposed to purchasing pharmaceuticals prepared under strict and sterile manufacturing practices, careful attention must be paid to precise measuring and aseptic procedures. This will prevent an adverse outcome when the compound is used in animals such as inadequate anesthesia, overdose, toxicity, or infection.

Preparation of Avertin

1. A concentrated (100%) **stock solution** of Avertin is prepared by mixing 5 g of 2,2,2-tribromoethanol with 5 ml of tertiary amyl alcohol (Sigma, Aldrich). Shake or stir gently until the solid is dissolved. The

stock solution is light sensitive and evaporates rapidly. Do not leave the bottle open longer than is necessary. Label, date, and refrigerate (4°C) in tightly sealed, dark amber or foil covered bottle. Yellowing of the solution indicates toxic degradation products and the stock must be replaced. Solutions with any crystal formation or precipitate must be discarded. ***Unused stock solution refrigerated at 4°C should be discarded after 6 months.*** Alternatively, the stock solution can be made and aliquoted into 1 ml snap cap tubes. The tubes containing the stock solution are then stored, wrapped in foil (to protect it from the light,) under an inert gas (nitrogen or argon) at -70°C. ***Stock solution stored frozen at -70°C under inert gas is stable indefinitely.*** Whether refrigerated or frozen, each tube must be labeled with the contents, preparation date, name of the preparer and, if refrigerated, the date of expiration. Stock solutions should be prepared in a fume hood while wearing gloves.

2. A **working solution** of Avertin is prepared by diluting 0.1 ml of the stock solution to 1.25 % in 7.9 ml isotonic saline (PBS), sterilize by filtration (0.2-micron filter) into a sterile injection vial. Label, date, and refrigerate when not in use. NOTE: When diluting the stock alcohol mixture with some commercially available complex phosphate buffered saline solutions, precipitation of the tribromoethyl alcohol may occur. This is due to the presence of calcium and/or magnesium in the PBS. To avoid this, check that the PBS you use is simple sodium phosphate buffered saline (0.9 %) and does not contain calcium and/or magnesium or use the following Tris buffered saline solution (0.9 % sodium chloride, 1mM Tris, pH 7.4, 0.25 mM EDTA). If there is crystallization or a change in color of the Avertin solution; it MUST NOT be used and should be disposed of properly. ***Working solutions are kept in the dark at 4C° and utilized within thirty (30) days of preparation.***

Use of Avertin

1. The working solution is administered intraperitoneally at 0.4-0.8 ml/mouse, (approximately 0.2ml/10 grams of body weight). Note that inadvertent intravenous injection will cause death within minutes. It will take about five minutes for the mouse to become fully anesthetized. An additional 0.05-0.1ml can be given to effect, allowing sufficient time after administering the additional anesthetic to observe the effect. Note that the effective dosage is dependent upon the weight of the mouse. Older animals, those with more body fat, or lactating animals will need a higher dose for complete anesthesia.
2. Verify anesthesia by confirming a reduced respiratory rate and lack of response to gentle pinching of the footpad (look for a lack of a withdrawal reflex).
3. Perform required surgery within five to ten minutes. The mouse will remain anesthetized for approximately 30-60 minutes. Supplemental dosing is not recommended.
4. After surgery, the mouse should be placed under a heating lamp or on a heated surface to prevent hypothermia. The animal should be observed post- surgically until it returns to consciousness.
5. Monitor the animal daily for several days for alertness, movement, and feeding. If any adverse effects of the surgery are observed, contact the Animal Facility or IC veterinarian for assistance or terminate the experiment by the method of euthanasia indicated in the ACUC approved protocol.

Stability of Avertin

1. Tribromoethanol may be transformed into toxic byproducts when exposed to light and temperatures above 4°C. Tribromoethanol properly formulated, filtered, and stored below 4°C in aliquots should assure the suitability of the anesthetic for routine use in mice.
2. Working solutions are kept in the dark at 4° C and utilized within thirty (30) days of preparation. Working solutions must be labeled with the expiration date of the solution.
3. When a bad stock of this compound is used, Avertin may induce lethality and/or peritoneal damage. If animals do not recover rapidly from the surgery or appear listless or die from non-experimentally induced procedures after several days to a week, then the tribromoethyl alcohol is suspect. In this

case, order a fresh bottle and make a new stock of Avertin.

References

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