

Concept Paper: Alternative Methods for Evaluating Locally Acting, Non-systemically Absorbed Drugs in Canine Disintegrating or Chewable, Single Layer Combination Drug Products.

INTRODUCTION

Background:

Whether dealing with human or veterinary drug products, the optimal method for evaluating the bioequivalence of two formulations is using comparative blood level trials. These studies are based upon an assumption that the drug enters the systemic circulation to reach its site of action. The blood level concentration versus time profiles that result from product administration, irrespective of route by which it is given, reflect the solubility of the active pharmaceutical ingredient in the biological environment, the permeability of the drug through biological barriers, and the effect of the formulation both on the rate and extent of drug release from the pharmaceutical product. For oral dosage forms, formulation can also influence the permeation of the active pharmaceutical ingredient across the enterocyte intestinal barrier.

Efforts to assess product bioequivalence are far more challenging when the drug is not systemically absorbed or when it acts both locally and systemically. For many years, the only study option in veterinary medicine has been the use of terminal clinical endpoint bioequivalence trials. However, these investigations can be more difficult to conduct because of the impact of potential challenges such as the inherent variability in drug response, the potential dependence of the product equivalence determination on study condition (e.g., duration of the infection and the integrity of the host immune system), and the need to include a large number of study subjects.^{1,2}

In keeping with the goals of reducing, replacing, and/or refining (the 3 R's) animal use (<https://www.nal.usda.gov/awic/animal-welfare-act>), the US Food and Drug Administration (FDA) Center for Veterinary Medicine (CVM) is engaged in efforts to identify alternatives to clinical endpoint bioequivalence trials for evaluating the bioequivalence of products containing locally acting drugs. It is essential that any alternative approach enable drug sponsors and the CVM to determine, with confidence, whether a test and reference formulation are bioequivalent. While state-of-the-art technologies are now available that can facilitate FDA/CVM Office of New Animal Drug Evaluation's (ONADE) efforts to address this unmet need (whether for topical formulations such as ophthalmic preparations, Type A medicated articles, or canine tablets containing both systemically absorbed and locally acting active pharmaceutical ingredients), validation of these proposed alternative pathways is needed before they can be used to support regulatory decisions.

The current proposal describes a study to validate use of an alternative approach for comparing canine oral dosage forms containing both locally and systemically acting active pharmaceutical ingredients. The results of this study will impact pre-approval generic drug applications and the evaluation of post-approval formulation changes for innovator and generic drug products. We hypothesize that through a combination of understanding the drug and formulation characteristics, a confirmation of product blood level bioequivalence in the animal (*in vivo*) for the systemically available active pharmaceutical ingredient, and the comparison of test and reference product dissolution characteristics under a range of *in vivo*-relevant conditions, the bioequivalence of the test and reference formulations can be evaluated for the systemically and non-systemically available active pharmaceutical ingredients. This would ultimately greatly reduce the numbers of dogs euthanized for the purpose of clinical endpoint bioequivalence trials.

ANTICIPATED OUTCOMES

CVM will publish the results of this study. Based on our overall investigational plan, two manuscripts are anticipated: a) the results of the *in vivo* and *in vitro* assessments; b) product comparisons based upon the *in silico* fitted estimates of *in vivo* dissolution versus the *in vitro* dissolution data.

If this investigation supports the use of this alternative approach for evaluating product bioequivalence of the non-systemically absorbed active pharmaceutical ingredient contained in a single layer combination oral dosage form for dogs, following publication of these manuscripts, a CVM guidance for Industry (GFI) will be drafted for public comment. The draft guidance will describe the proposed alternative bioequivalence pathway for non-systemically absorbed active pharmaceutical ingredients contained in canine combination product oral dosage forms when at least one of the active pharmaceutical ingredients is systemically absorbed.

We anticipate benefits to drug sponsors, the public, and animals.

STUDY DESIGN

Investigational Constraints:

Several critical considerations were integrated into the development of the proposed study:

1. The results need to provide a scientifically sound assessment of the proposed hypothesis that through a combination of drug and formulation understanding, a confirmation of product *in vivo* blood level bioequivalence for the systemically available active pharmaceutical ingredient, and the comparison of test and reference product dissolution characteristics under a range of *in vivo*-relevant conditions, we can be confident in our assessments of product bioequivalence for the systemically and non-systemically available active pharmaceutical ingredients.
2. Drugs used in the study need to have previously been approved for use in combination, allowing CVM to leverage the extensive characterization of these products that were performed as part of the approval process.
3. Dosages of the drug contained in the respective formulations should not exceed levels for which CVM has confirmation of their safety to assure the relevance of the collected information.
4. Drugs selected must be systemically absorbed, thereby proving a benchmark against which our bioequivalence predictions can be evaluated.
5. The number of dogs used in the study should be sufficient to draw conclusions without compromising the integrity of the study.
6. Animal experimentation should be consistent with the types of studies typically conducted to support a human *in vivo* bioequivalence assessment to allow leveraging of the experience in human medicine.
7. The study must be non-terminal, as there are no scientifically valid reasons to do otherwise.
8. The dogs must be retired following completion of the blood level study and the study must not in any way render them in a condition that causes them to be unacceptable for future adoption.

Drug Selection:

Systemically absorbed drugs were selected based on their markedly different physicochemical and pharmacokinetic (PK) characteristics (to provide a rigorous testing of our proposal) and on their prior FDA/CVM approval for use in combination for dogs. To that end, the selected drug combination is ivermectin (IVM) and praziquantel (PRZ).³ IVM is a large highly lipophilic molecule which is insoluble in water (0.0040 mg drug dissolved per /mL water). It is expected to have good passive membrane permeable (based upon

the calculated log of the partitioning of the drug between octanol and water, Log Pow of approximately 3.22⁴, a conclusion that is consistent with its experimental extrinsic membrane permeability^{4a}. These data suggest that the large size of IVM does not impair its uptake by passive diffusion across biological membranes. In contrast, PRZ is far more hydrophilic (although classified as low solubility, its aqueous solubility = 0.1 mg/mL), with a slightly lower cLog P (3.36, which still falls within the bounds defining highly permeable compounds).⁵ IVM tends to be slowly absorbed (Time to maximum plasma concentrations, Tmax, is about 4 hrs), with a long residence time in the body (can be detected for over 20 days; estimated terminal elimination half-life, T½β, = 3-4 days).⁶ In contrast, PRZ is rapidly absorbed (about 0.5 to 2 hrs postdose), and with a far more rapid elimination half-life (<2 days).⁷ Both compounds are contained within the approved Iverhart Max (Virbac) for dogs.

***In Vitro* Study:**

Formulation development and *in vitro* testing of the experimental formulations will be outsourced.

A range of single layer tablet formulations will be developed, varying formulation and/or processing characteristics (tablet hardness). A battery of *in vitro* dissolution tests will be conducted across a range of media (consistent with the soon to be released <USP 1236> Solubility Measurements) on each formulation. Additional surfactant may be added to some of the media if deemed appropriate. Based upon the *in vitro* release specifications targeted by ONADE scientists to test the applicability of this alternative bioequivalence approach, three of the formations will be selected for the *in vivo* trial such that:

- If Formulation A = the “reference”
- Then as compared to Formulation A, Formulation B will exhibit similar *in vitro* release of PRZ but different release of IVM.
- As compared to Formulation A, Formulation C will exhibit similar *in vitro* release of IVM but different release of PRZ.
- Formulations C and B differ in terms of *in vitro* release of both active pharmaceutical ingredients.

The amount of IVM and PRZ included in the experimental formulations will be within the bounds of what is approved for use in dogs.

***In vivo* Study:**

The *in vivo* component of this study will be conducted at an FDA facility.

- **Design considerations:**

The study will be conducted as a three treatment, three period three sequence crossover investigation from which three sets of pairwise comparisons can be obtained (Formulation A vs B, A vs C, B vs C).⁸ The design of the study will be as follows:

Sequence	Period 1	Period 2	Period 3
1	Treatment A	Treatment B	Treatment C
2	Treatment B	Treatment C	Treatment A
3	Treatment C	Treatment A	Treatment B

For each set of comparisons, two separate sets of assessments will be generated:

- 1) Assuming the PRZ is the systemically available drug and IVM is not systemically available, blood level bioequivalence will be determined for PRZ and formulation comparison for IVM will be based upon a series of *in vitro* dissolution profiles. *In vitro* dissolution comparisons will be generated using 12 tablets of the “test” and “reference” formulations. Profiles will be compared based on the F2 metric, where F2 is a mathematical algorithm used to test *in vitro* dissolution profile comparability (i.e., the “similarity” factor).⁹ Profiles will be generated under the range of conditions consistent with canine gastric and intestinal fluid composition as described in the soon to be released USP general chapter <1236>.
- 2) Assuming the IVM is the systemically available drug but PRZ is not systemically available, blood level bioequivalence will be determined for IVM and formulation comparison for PRZ will be based upon a series of *in vitro* dissolution profiles as described above.

Using this study design, the investigation will result in 6 sets of *in vivo* bioequivalence comparisons:

- Trt A – PRZ vs Trt B – PRZ
- Trt A – PRZ vs Trt C – PRZ
- Trt B – PRZ vs Trt C – PRZ
- Trt A – IVM vs Trt B – IVM
- Trt A – IVM vs Trt C – IVM
- Trt B – IVM vs Trt C – IVM

The same set of 6 *in vitro* bioequivalence comparisons will be generated.

From the relationship between observed (*in vivo* bioequivalence study outcomes) versus predictions (bioequivalence assessments based on comparative *in vitro* dissolution profiles), we will determine if we successfully predicted product equivalence/inequivalence for the non-systemically available active pharmaceutical ingredient. Even if the active pharmaceutical ingredient designated as systemically available is found not to be bioequivalent based upon the blood level data, the *in vitro* comparison will nevertheless be conducted to ascertain if the battery of *in vitro* dissolution tests plus the *in vivo* bioequivalence data could identify inequivalent formulations and if the *in vitro* dissolution tests correctly identified the equivalence or inequivalence of the “non-systemically available” drug.

Upon arrival, dogs will be housed for socialization for approximately three months prior to the first drug administration. This will reduce animal stress and will facilitate the handling, dosing, and sample collection during the study. Following the final dose, the dogs will be in quarantine for two weeks to ensure their health and readiness to be retired for adoption to their forever homes.

To capture the profiles of the two active pharmaceutical ingredients, blood samples will be collected from each animal pre-dose and at 1, 2, 3, 4, 5, 6, 8, 12, 24, 72, 120, 168, and 240 hr post-dose. To avoid the potential for a carryover of IVM in the plasma, a minimum of a 4-week washout interval will separate each dosing period.¹⁰ The dogs will be at the facility for three months prior to the study and two weeks after the *in vivo* phase of the study. The *in vivo* portion of the investigation will cover a duration of nine months.

To support this *in vitro* bioequivalence study, an efficient, selective and sensitive bioanalytical LC/MS/MS method will be developed and validated for simultaneous determination of IVM and PRZ in beagle dog plasma.

- **Estimating subject (subj) number:**

Although the investigation is conducted using a three-period (Per), three-sequence (Seq), three-treatment (Trt) crossover, comparisons will be pairwise such that at any given time, one period per animal is not included in the assessment (collapsed model). In so doing, we effectively have 3 sets of analyses (A vs B, A vs C, B vs C), each containing animal data associated with a three period, three sequences 2 treatments (<https://analytics.ncsu.edu/sesug/2004/SD04-Yarandi.pdf>).

When determining the number of dogs needed in this study, it is necessary to estimate the within subject variability associated with the treatment comparison. Without pilot data, we relied upon the published literature to inform the sample size estimation. Based on information from studies of IVM and PRZ in dogs, we assumed that the estimated within dog variability for IVM was greater than or equal to that of PRZ. The within dog variability of reported for IVM was 23% based upon blood level data generated using a radioimmunoassay.¹¹ Allowing an expectation of slightly greater variability within dog, we set the estimated variability across the pairwise comparisons as 25%.

The DF that will be that associated with each pairwise comparison (Seq minus 1 = 2; Per minus 1 = 2; subj(Seq) – 3 = “to be determined”; and Trt -1 = 1). Using a spreadsheet presented during the 5/12/2015 public meeting, we concluded that 8 dogs per sequence is the minimum needed to achieve 90% confidence limits of 0.80 to 1.25 with 80% power if the ratio between observed treatment parameter means is close to 1 and the estimated variability is realized. Therefore, we conclude that the minimum number of dogs needed for this investigation is 24.

However, experience from similar studies, along with scientific judgement, necessitates preparedness for situations that might require one or more dogs to be removed from the study prior to study completion. As such, there would be the very real risk of invalidating the study outcomes. To preclude that possibility, we have determined that an additional animal needs to be added to the study.

Since we will be using three-period, three-sequence, three-treatment crossover design for this study, one cannot add just a single dog to the overall study, as this would result in an unbalanced study design. Therefore, we will need to add three additional dogs, one per sequence, raising the overall number of dogs to 27, with 9 dogs per sequence.

- **Bioequivalence Assessment:**

The first step is to confirm the absence of a statistically significant sequence effect (using animals within sequence as the error term for testing that main effect). All data for a given active pharmaceutical ingredient will be used in this first assessment, thus having a degrees of freedom (DF) that are consistent with the study as designed. The model degrees of freedom (DF) for the ANOVA will equal Seq minus 1 = 2; Per minus 1 = 2; subj(Seq) – 3; and Trt -1 = 2.

For the pairwise bioequivalence comparisons (and the confidence intervals that will be used to evaluate product bioequivalence), an ANOVA will be run using only the treatments being compared. In that case, the DF used for the confidence interval determination is $2*(3n-2)$. Accordingly, if we have $n = 8$ per sequence, the DF used for the confidence interval estimate would be $2*16 = 32$.

***In vivo* Mechanistic Model:**

Physiologically based pharmacokinetic (PBPK) models are powerful tools for integrating diverse sources of information to generate predictions regarding drug and formulation characteristics that will exert the greatest magnitude of influence on *in vivo* dissolution, absorption, and oral bioavailability. These tools provide an opportunity to address questions that would otherwise be impossible to answer without an

extensive array of *in vivo* and *in vitro* studies. Mechanistic PBPK models provide an opportunity to integrate gastrointestinal transit time, absorptive surface area, membrane permeability, transporter activities, local drug metabolism, luminal fluid contents, drug attributes, and drug product information with *in vitro* dissolution data to predict performance of the drug in the live animal.

The currently proposed investigation offers an opportunity to explore the use of mechanistic absorption models to predict *in vivo* dissolution based upon drug physicochemical characteristics, product formulation, and *in vitro* dissolution data generated under a range of media and apparatus speeds. Knowing the site in the intestine being targeted for drug delivery, it can be used to estimate the magnitude of formulation-induced altered *in vivo* dissolution necessary for products to no longer effect equivalent total drug exposure at the local site of action. It can also be used to explore the bias in product relative bioavailability conclusions that could be associated with data derived across a range of *in vitro* dissolution conditions. CVM has previously published an example of how *in silico* PBPK models can be used to explore intestinal drug dissolution characteristics in humans and in dogs.¹² However, the focus of that research was on modeling intestinal exposure based upon blood level data (a middle-out approach).^{13,14}

Under the conditions of the current research project, we will predict the impact of varying product dissolution characteristics based on the *in vitro* data generated across a range of formulations. We will designate one API in the combination tablet in turn as being “not systemically available” while the other is handled as being “systemically available”. The blood level data generated for the designated systemically available drug in the combination tablet, the physical and chemical characteristics of the two API’s in our experimental formulations, and the *in vitro* dissolution data generated for both tablet components will support our prediction of the likely gastrointestinal location of dissolution for the “not systemically available” API. The accuracy of our predictions will be assessed by fitting the intestinal dissolution profile to the observed *in vivo* blood level data generated during the *in vivo* PK portion of this study (a form of external validation). Within the framework of this study design, the validation process will also help to identify any additional information that needs to be collected to improve our model predictions. This process will be repeated for the three formulations, with both API’s sequentially designated as the “non-systemically absorbed” component. By comparing the fitted versus predicted *in vivo* dissolution curves, we can assess whether or not our proposed method for evaluating the similarity or differences in the “test” versus “reference” product based upon blood levels for one component and comparative dissolution generated on both APIs can provide an accurate determination of product relative bioavailability.

If the results of the *in vivo* blood level product comparisons support the accuracy of our *in vitro* and model predictions, this study will be used to demonstrate the capability of these kinds of studies to support *in vivo* bioequivalence for combination tablets when one of the components is systemically absorbed but the other(s) acts only locally. In so doing, we will avoid the need to artificially infect dogs to confirm the equivalence of the non-systemically absorbed component(s) of the of a test and reference formulations. While we anticipate the use of this alternative procedure to support generic drug products, it is important to note that use of this mechanistic modeling tool may be particularly valuable to innovators (who have the underlying clinical data), especially when differences in *in vitro* dissolution profiles are observed.

By employing mechanistic absorption models, this study can provide a roadmap that sponsors can use for determining the likelihood that observed differences in *in vitro* dissolution profiles will impact *in vivo* effectiveness for a locally acting compound. In this case, the models can provide the predictions necessary to determine the relationship between *in vitro* profile deviations vs local gut exposure, to optimize the *in vitro* procedures used to characterize *in vivo* product performance, and by comparing results with their existing clinical data, define the magnitude of variability in intestinal exposure that would be without therapeutic relevance.

CONSEQUENCES OF DOING THE STUDY

Benefit to Drug Sponsors:

Innovators: This highly efficient, time effective and cost-effective approach will encourage innovator firms to modify existing formulations in a manner consistent with a desire to reduce animal numbers. Furthermore, this approach will enable sponsors to address Scale-up and post-approval changes (SUPAC) Level 3 changes¹ that currently would necessitate both blood level relative bioavailability trials as well as clinical endpoint bioequivalence trials. Implementation of the proposed *in vitro* dissolution model can be expected to save cost in the development of necessary data and in the use of animals.

Generics: This highly efficient, time and cost-effective approach will help encourage sponsors to develop generic formulations, providing an economical alternative for much needed canine therapeutics. Implementation of the proposed *in vitro* dissolution model can be expected to save cost in the development of necessary data and in the use of animals.

Benefit to the Public:

There will be greater availability of innovative new animal drug formulations and of generic formulations of brand name products that have since gone off patent. Additionally, many of the non-systemically absorbed drugs used in canine medicine are indicated for the treatment of intestinal parasites. Parasitic infections can often be transmitted between animals and humans. Insuring that dog owners have cost effective ways of dealing with these infections in their pets (prevention and/or cure) provides important positive public health consequences.

Benefit to the Animal:

Validation of the *in vitro* dissolution model will provide a useful alternative to conducting clinical trials for the bioequivalence of non-systemically absorbed animal drugs. It is anticipated that this will result in fewer animal studies being needed. When considering where this tool can be used, which includes future generic products, post-approval chemistry and manufacturing changes (generic and innovator), and those instances where innovator pre-market formulation modifications will necessitate the use of artificial infection clinical endpoint bioequivalence studies to bridge to existing safety and/or effectiveness data, we estimate that the availability of the proposed *in vitro* dissolution model will likely result in saving more than three quarters of the animals otherwise required. In a 2-5 year period, that equates to approximately several hundred animals. This is a substantial reduction in the terminal use of dogs.

RISKS AND MITIGATION PLAN

The risks of this research must be considered from two perspectives: the study itself and the well-being of the dogs while resident at the FDA laboratory.

¹ Immediate release scale-up and post approval level 3 changes are those changes that are likely to have a significant impact on formulation quality and performance.

Guidance for Industry. Immediate Release Solid Oral Dosage Forms. Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, *In vitro* Dissolution Testing, and *In vivo* Bioequivalence Documentation. FDA CDER 1995.

From a Study Perspective:

- a. *What is the likelihood of tablet failure to perform?* This concern is associated with the potential for problems with the manufacture of the tablets. To mitigate this risk, the clinical lots will undergo a thorough battery of in vitro characterization (physicochemical characteristics and in vitro dissolution under several conditions) before being administered to the dogs. In this regard, all study medications will be made to standards used for Phase 1 human clinical trials (<https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070273.pdf>)
- b. *What happens if there are occasions where a tablet coming from a batch that has met Quality Control specifications fails to be absorbed?* This is a potential problem that can occur whenever a drug is orally administered. For example, if the dose happens to be administered during the propagation of a housekeeping wave (as observed in humans and dogs), that dose may exhibit near zero oral bioavailability. The advantage of this study is that in that situation, we would anticipate that neither component would be absorbed. However, if one API is absorbed normally while the other is barely absorbed, we would need to evaluate whether this possible problem was identified during lot in vitro characterization. Failure to identify such a problem would imply that our in vitro BE approach may contain fatal flaws as currently devised. This is all part of the information that we seek to obtain through the conduct of this investigation.
- c. *Will dog drop-outs and the corresponding loss of study power result in the need to repeat the study?* We have estimated the number of dogs to achieve the power needed to meet traditional confidence interval criteria for identifying bioequivalent products. However, even under ideal conditions, a larger within-animal variability than we initially anticipated is possible. Similarly, a need to remove a dog prematurely from the study could lead to a study with insufficient power to declare formulations bioequivalent. In either case, it is essential to distinguish the objectives of this study from what would be required of a regulatory submission to demonstrate product bioequivalence. In this proposed investigation, we are generating the data needed to determine if in vitro dissolution plus blood levels from the systemically absorbed component can accurately predict in vivo product equivalent/inequivalence for the non-systemically available drug in a combination drug product. Thus, if there is inadequate power to confirm equivalence with the observed data alone, we have the option to employ Monte Carlo methods to further our data exploration, utilize in silico models to help us examine the in vitro- in vivo relationships, or utilize other statistical approaches to make this assessment in the absence of the targeted study power (e.g., with the given ratio in treatment means and residual error, how many animals would be needed to declare the products as equivalent? Which formulations are the most variable? Was there evidence available from in vitro work that might have alerted us to this problem?). Irrespective of outcome, all findings will be considered important and could help identify questions that need to be addressed. We have addressed the potential for a dog's premature study removal by increasing the number of dogs that will be used for this study just above the minimum number calculated to provide the requisite information.
- d. *Would failure to adequately model the data negatively influence the relevance of this trial?* The *in silico* component of this study has the potential to be powerful, as it is used both to describe the likely in vivo dissolution profile for the various formulations and APIs and to explore the utility of this tool within a regulatory framework. Should we find that we are unable to overcome the magnitude of model misspecification that would enable us to effectively use a middle-out approach to predict in vivo dissolution characteristics, it would not negate the value of the information derived from the observed study results. Such an outcome would also alert us to the challenge of trying to employ these in-silico models as a tool for supporting out in vitro product BE assessments for locally acting drug products.

From the Perspective of Dogs while in Residence at the Study Facility

The risks associated with the research phase of this study that need a mitigation plan principally revolve around issues associated with adverse events that impact the animals' ability to remain on the study. The risks to the overall study outcomes associated with animals prematurely leaving the study have been addressed above.

The risks can be summarized as all study-required procedures and activities performed by study personnel that might negatively impact a dog's health. This includes both procedures and activities that directly support the acquisition of blood samples necessary to determine the concentrations of the two drugs and all other activities associated with the care and housing of the animals while at the study facility.

- a. Prior to the dog's arrival, the proposed animal use protocol will be reviewed by the facilities Institutional Animal Care and Use Committee to ensure compliance with requirements in the Animal Welfare Act and to ensure consistency with Public Health Service (PHS) Policy. This review will also address issues of animal pain and distress associated with their involvement in this study. The dogs will be under the constant care of an Attending Veterinarian for the study facility. All dogs will undergo a health exam by the Attending Veterinarian at the time of their arrival at the study facility to ensure their suitability to be placed on the animal use protocol. The veterinary staff will review the records which accompany the dogs to the study facility to ensure all necessary vaccinations have been given to the dogs to ensure their continued good health prior to formal study initiation. A detailed electronic daily observation tracking database will be used by all study personnel, including the veterinarians and animal care staff, to record all daily observations of the dogs. This database will facilitate review of an animal's health status by the veterinary staff.
- b. The dogs will be extensively socialized prior to initiation of the drug- administration phase of this study. Socialization improves the human-dog interactions, reducing the stress the dogs would otherwise exhibit upon human interactions. The extensive socialization schedule that will be used in this study also facilitates the human-animal connection and provides a more frequent set of observations on the overall health of the animal. The extensive socialization processes used in this study will facilitate the eventual placement of these dogs in their forever homes.
- c. All study personnel and animal care staff will be trained by the Attending Veterinarian on the types of observationally derived signs that could indicate pain or distress in the dogs.
- d. All observations by the animal care staff and other study personnel will be entered into the electronic database for review by the Attending Veterinarian.
- e. As part of the socialization process, the dogs will be acclimated to the types of procedures, including handling, that will be used administer the drugs and collect blood from them. In addition to human-dog interactions, socialization will also include toys and treats. The dogs will also be given the opportunity for direct interaction and play with the other dogs that are part of this study. The net result of the entire socialization process is that the animals will interact with study personnel at least 4 to 5 times per day. The success of the socialization process will be determined using a qualitative assessment scoring guide. The drug-administration phase will not commence until all animals achieve an assessment score of 5; Animal approaches you without the need to present treats.
- f. All dogs will be individually housed, with their own food and water bowls. All dogs will be housed indoors in a climate-controlled facility. Each pen is 28 square feet. The minimum recommended space requirements for dogs of the size to be acquired for this study is 8 square feet. The dogs will initially be singly or pair-housed to facilitate acclimation to the new facility, depending on how the dogs are housed at the breeder's facility. Special care and heightened observations will be performed the first time the dogs can freely interact with each other. Dogs that initially cannot socially interact with other dogs will be subsequently released individually for the free time running in the study facility. Efforts will be made to socialize the dogs to overcome this inability to interact with other dogs.

- g. At the conclusion of the in vivo phase of this study, the dogs will be prepared for their transition to pets by halter-walking the dogs outside on various surfaces, including grass.
- h. By employing these socialization approaches, we will reduce the overall stress on the dogs, which in turn mitigates one of the primary factors that can negatively impacts their health. Furthermore, the socialization process will dramatically facilitate the collection of blood from the animals. Experience with other species has demonstrated that socialization and the use of treats, along with the use of indwelling catheters, eliminated the need for physical restraint during blood collection procedures. A by-product of the socialization of the animals is that there is a dramatically reduced risk of injury to the staff members collecting the animals blood
- i. The dogs will be given additional health exams between each drug treatment period to ensure that they are still healthy and suitable to remain on the animal use protocol.
- j. The blood draws obtained during the early time periods after drug administration will be collected from an in-dwelling catheter to minimize the stress to the dogs of repeat blood draws. Insertion of the indwelling catheters will be performed by the OR veterinary staff and/or the veterinarians working on this study.
- k. Should a dog need to be removed from further participation in the study, they will be returned to good health prior to being retired for adoption with the rest of the dogs on this study.

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