Avoiding the Pitfalls of Preanalytical Variables in Animal Health Studies

Introduction
Clinical pathology data are routinely collected during animal health studies in order to aid in the evaluation of a compound's toxicity profile. While these data are useful for the identification and monitoring of potential toxicities, their value is dependent on the analysis of quality samples. Clinical pathology test results can be affected by factors occurring prior to sample analysis (preanalytical), during sample analysis (analytical), and following sample analysis (post-analytical). The potential impact of preanalytical variables, in particular, can be easily overlooked at the time of data interpretation and it is essential to consider these effects during study design and sample collection in order to avoid the generation of inaccurate or misleading data and to prevent erroneous attribution of clinical pathology changes to compound administration.

Preanalytical variables important in animal health studies can be broadly separated into four categories, including physiologic factors pertaining to the animal, factors related to study procedures, factors related to sample collection and handling, and factors related to sample processing in the clinical pathology laboratory, and each of these will be discussed in the following sections. Additional discussions of the impact of preanalytical variables on clinical pathology data in animals can be found elsewhere.[1-5] A common theme that will be presented is that, although not all sources of preanalytical variability can be avoided in all cases, consistency in study procedures among experimental groups is essential to avoid confounding effects that could lead to inaccurate data interpretation.

Physiologic Factors Related to the Animal

Preanalytical variables related to the physiologic state of the animal include factors such as age, sex, breed or strain, reproductive status, and general health status. Above all, it is necessary to ensure that, within the confines of the target animal population, these factors are randomised among experimental groups to ensure relatively equivalent distribution. Each of these factors has the potential to impact clinical pathology endpoints, and similarity across groups helps to avoid the occurrence of a numerical difference in the data that is due to normal physiology but could be misinterpreted as being a compound-related change. For example, the enzyme alkaline phosphatase can be a marker of hepatobiliary toxicity, but is also produced by osteoblasts in bone and values are higher in young animals associated with normal skeletal growth. Failure to age-match experimental groups could result in incorrect identification of a potential hepatobiliary effect based on higher serum alkaline phosphatase activity, when in fact the increased values may be due to a younger cohort of animals. Other clinical pathology variables, such as red blood cell count and albumin concentration, are lower in younger animals and gradually increase with age until adulthood. Differences in several haematology and clinical chemistry values are common between males and females of most animal species, and if group mean data are not evaluated separately from each sex, it is imperative that the number of males and females is equivalent across groups to avoid sex-related differences as a confounding variable.

In addition to age and sex, different breeds or genetic lineages of animals may exhibit differences in some clinical pathology parameters. In general these differences are minor and unlikely to affect interpretation of group mean values, but may have an effect in field studies if a certain breed is over-represented. In target animal safety studies, mostly for consumption animals, breed differences need to be carefully considered as significant differences in clinical pathology parameters can occur. For example, physiological differences between dairy and beef cattle or between broiler chickens and laying hens will translate into great variability in some parameters. These considerations further underscore the need for randomisation during group assignment.

Other physiologic factors are intertwined with the experimental design and include fasting status and diurnal variation in the concentration of certain analytes. Changes in some clinical chemistry parameters, such as triglycerides, can be affected by food consumption, and post-prandial lipemia (accumulation of lipids in serum) can affect the accurate measurement of parameters that depend on light refraction, such as haemoglobin. Any changes in fasting status due to veterinary intervention or other circumstances should be documented in the study records so the potential effects of these changes can be weighed during data interpretation.

Consideration of the timing of sample collection is also important due to diurnal variation of some analytes. As a general practice, samples should be collected at approximately the same time of day (early morning, late morning, etc.) for each group and on each day of collection. This is most critical for hormones, which frequently have a distinct and predictable diurnal pattern. Hormones serve as biomarkers for endocrine organ function, and inconsistent timing of sample collection could potentially lead to discrepant values that may suggest a functional effect related to the administered compound.

Factors Related to Study Procedures

There are a number of study procedures that may affect clinical pathology data and, although it is not always possible to avoid these effects, it is essential to ensure that the same scope and duration of study procedures are carried out for all groups.

Transport, handling, restraint, blood collection, and other procedures can contribute to excitement or fear (induced by epinephrine) or physiologic stress (induced by glucocorticoids). Excitement and fear are associated with increased heart rate and blood pressure, which contributes to increased measured neutrophil and lymphocyte counts, often reflected as an increased total white blood cell count. Physiologic stress, on the other hand, is generally associated with increased neutrophil and decreased lymphocyte

and eosinophil counts due primarily to the effects of glucocorticoids on leukocyte trafficking, also commonly reflected as an increased total white blood cell count. Both processes may also occur concurrently. Because of these potential effects, study procedures that may be expected to cause excitement and/or stress should be comparable across groups to avoid inducing these leukocyte changes in a single group. The effects of excitement and/or stress should also be taken into account when comparing to baseline data. It is common to see leukocyte changes associated with excitement at the first blood collection, as animals are being acclimated to study conditions and procedures. This may manifest as higher total white blood cell (neutrophil and lymphocyte) counts during the prestudy phase and lower cell counts during the dosing phase (after animals have been acclimated). In addition, fear and/or excitement at the time of blood collection may cause splenic contraction, resulting in elevated red blood cell mass parameters. If allowable within recommended blood collection limits for the animal size, the collection of two baseline samples from each animal is often helpful in that it allows for more thorough interpretation of these potential changes during the prestudy period and is useful in establishing the biologic variability in clinical pathology variables for each animal.

Similarly, the use of anaesthesia during blood collection should be also be consistent as alert animals typically have higher measured total white blood cell and red blood cell counts compared to anaesthetised animals due to excitement. Changes in other measured values may also be altered in anaesthetised animals due to the biologic effects of the anaesthetic and changes in heart rate and respiration.

Restraint, handling, and intramuscular injections often cause mild to moderate increases in the serum activity of enzymes from skeletal muscle, most notably creatine kinase. Again, although these procedures are frequently unavoidable during the conduct of a study, standardisation of procedures is critical to avoid inconsistent effects among experimental groups. Activity level may influence some clinical pathology parameters due to muscular activity and excitement, possibly creating differences in total white blood cell counts from samples taken at rest.

Previous blood collection is a common preanalytical variable for which consistency is essential. Collection of moderate blood volumes for clinical pathology and other evaluations results in a transient decrease in circulating red blood cell mass that is replaced by newly-formed cells within several days in healthy animals. For this reason, it is common to see mild decreases in red blood cell mass following previous blood collections; however, the association of these changes to venipuncture is generally concluded based on a similar magnitude of change in other groups (including a relevant control group). Therefore, it is necessary to ensure that the intended frequency and volume of blood collection is consistent across animals in all groups, so that direct comparisons can be made and incorrect identification of a compound-related decrease in red blood cell mass can be avoided.

Factors Related to the Sample

Samples for clinical pathology evaluation should be collected in the same manner from all animals, including site and technique. Blood collection site may cause variability in haematologic parameters, so having a consistent venipuncture site is important to avoid this additional variability in the data. It is also critical to ensure that the correct matrix, tube, and blood to anticoagulant ratio are being used for the intended analysis. In general, haematology evaluations are typically performed on samples anticoagulated with ethylenediaminetetraacetic acid (EDTA), coagulation testing is generally performed on samples anticoagulated with sodium citrate, and clinical chemistry samples are performed either on non-anticoagulated serum samples or on lithium heparin-anticoagulated samples. Lithium heparin can be used for both clinical chemistry and haematology evaluation in birds. In addition to using the correct anticoagulant, attention should be paid to ensuring a proper blood to anticoagulant ratio. This is most critical for citrated samples, which generally require a 1:9 ratio of anticoagulant to blood. Overfilling or, more commonly, underfilling the tube can cause erroneous results due to under- or over-citration of the resulting plasma, leading to alterations in coagulation test measurements that may be misinterpreted as a compound-related effect. Similarly, tubes containing EDTA should be filled to the specified tube volume as significant underfilling may cause sample dilution and lower measured blood cell counts. Using a tube with a volume appropriate for the size of the species is recommended to ensure that proper tube filling is achievable.

In our experience, less-than-ideal blood collection technique and sample handling are the most common reasons for erroneous laboratory results, particularly for haematologic evaluation. A quality sample is one which is collected by atraumatic venipuncture, transferred quickly to the correct tube, mixed well (for anticoagulated samples), and rapidly brought to the laboratory for processing. Traumatic venipuncture is most commonly due to multiple “sticks” or re-directions of the needle, which induce injury to vascular endothelial cells, release of procoagulant mediators, and activation of platelets, all of which promote clotting. Similarly, excessive negative pressure on the syringe plunger during sample aspiration can induce turbulent blood flow into the syringe, contributing to haemolysis and platelet activation. Animals of small weight, including young/juvenile animals, have small blood vessels that can easily collapse, making atraumatic venipuncture challenging. Cats and rodents are especially sensitive to excessive platelet aggregation (clumping) associated with traumatic sample collection, which are commonly causative of decreased measured platelet counts in these species.

Samples should be transferred to appropriately labelled tubes containing the correct anticoagulant (or lack thereof) immediately after collection, and anticoagulated samples should be well-mixed by 5-10 gentle inversions to ensure even distribution of the anticoagulant, as inadequate mixing often leads to clotted samples. Empty tubes containing anticoagulant should not be left uncapped for extended periods prior to blood collection (e.g. while materials are being set up or animals are being removed from cages) as the

Physiologic factors	Study procedures	Factors related to sample collection	Factors in the clinical pathology laboratory
Age Sex Breed or strain Reproductive status Health status Fasting or non-fasting Diurnal variation	Restraint/handling Blood collection Anaesthesia	Collection technique Anticoagulant (type and blood to anticoagulant ratio) Sample transport	Sample processing and storage Order of sample analysis

Table 1. Possible sources of preanalytical variability in animal health studies

small volume of anticoagulant is prone to evaporation. Samples should be brought to the laboratory as soon as possible, as most analytes are not stable at ambient temperature for more than a few hours, and delayed separation of serum from cells for biochemistry analysis can lead to inaccurate results for some analytes, such as glucose.

Contamination of samples is generally not a widespread issue, although the venipuncture area should be visually inspected for any potential debris that may contaminate the sample. Contamination of urine, however, can be more problematic in that voided samples are frequently collected due to logistical challenges associated with cystocentesis or urinary catheterisation in large groups of animals, and are prone to environmental contamination if collected from an open reservoir such as a cage pan. Contaminants such as faeces, food, cleaning products, and other substances may cause positive values for various urine test strip ("dipstick") assays, and therefore efforts to limit sample contamination of urine should be employed.

Factors in the Clinical Pathology Laboratory

In the clinical pathology laboratory, preanalytical variables that may affect the integrity of the data include sample handling and storage and the order of sample analysis. As previously discussed, samples should be analysed or appropriately stored as quickly as possible, as most analytes do not have long-term stability at room temperature. Once processed, samples should not be left exposed to ambient air as evaporation is possible and may lead to artifactual increases in some analytes. Additionally, if samples are to be stored prior to analysis, it is important to follow the storage specifications for the assays, per manufacturer guidelines and based on stability data generated during method validation studies. Samples for clinical chemistry analysis and coagulation testing are generally stored frozen if analysis is delayed. Haematology samples can generally be stored refrigerated for up to 24 hours prior to analysis, although this is not recommended on a routine basis due to *in vitro* cell degeneration, and an effort should be made to analyse samples as soon as possible after collection.

The order of sample analysis is an important consideration to prevent the introduction of artifactual differences in measured values among groups associated with differences in the time between collection and analysis. Randomisation of the order of sample analysis or round robin analysis (one animal from Group 1, one animal from Group 2, etc.) can be employed to ensure that there is not a large lapse of time between analysis of samples in one group relative to another group that may contribute to stability-related changes in measured analytes that might mask or be confused for a compound-related effect. If samples are collected in random or round robin order, this is usually sufficient to prevent potential block effects among groups and further randomisation of the order of analysis may not be necessary.

Conclusions

Preanalytical variables encompass factors related to animal physiology, study procedures, and sample collection and handling, and should be considered in depth during both study design and study conduct to reduce the potential impact of these variables on clinical pathology data generated as part of animal health studies. Although not all possible preanalytical variables can be avoided under all circumstances, it is critical to ensure consistency of study conditions and procedures among experimental groups to avoid confounding effects that may complicate the interpretation of compound-related changes in clinical pathology data.

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