ESTABLISHING DECISION LIMITS FOR THERAPEUTIC DRUGS DETECTED IN ANIMALS EXHIBITED AT LIVESTOCK SHOWS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2019

Major Subject: Biomedical Sciences

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ABSTRACT

Food animal species, such as cattle, sheep, goats, and pigs, are widely exhibited at stock shows and fairs across the United States. Animals are judged on phenotypical traits such as muscling, structural correctness, and frame-size. The level of competition is high, increasing the potential for illegal or unethical acts to gain a competitive advantage, such as doping. Anti-doping regulation in the livestock show industry often involves drug testing. Detection of therapeutic drugs at very low concentrations in approved animal species raises questions about current anti-doping regulations in exhibition animals. Therefore, the purpose of this research was to identify and address data gaps in the current understanding of drug testing and drug disposition, and to integrate those data with published data to propose an approach to standardizing anti-doping policies, particularly those related to therapeutic drug use.

To determine which drugs are most commonly identified in drug testing at livestock shows, a review of historical drug test results from a laboratory in Texas from 1999 to 2017 was performed. A total of 32,027 samples were tested during this period, of which 1,674 (5.2%) tested positive. Positive samples included a total of 42 different drugs and metabolites. Flunixin was the second most commonly identified drug.

Currently no nonsteroidal anti-inflammatory drugs (NSAIDs) are approved by the Food and Drug Administration (FDA) for small ruminants, but drugs such as flunixin and meloxicam are used in small ruminants. Additionally, urine is the sample of choice when drug testing exhibition animals but there is a gap in the scientific literature describing drug concentrations in urine of small ruminants. Therefore, pharmacokinetic studies were performed describing plasma and urine concentrations of flunixin meglumine and meloxicam in goats. Drug levels in urine

reached peak concentrations between 8 and 12 hours after dosing for both drugs. Urine concentrations for both flunixin and meloxicam fell below the limit of detection (LOD) of 0.5 ng/mL and 1 ng/mL, respectively, by 240 hours.

Last, observed and published data, PK/PD modeling results, and measurement uncertainty were integrated to propose a method for establishing decision limits for therapeutic drugs detected in urine from animals exhibited at livestock shows.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Fajt, for her guidance over the course of my graduate studies. I am grateful for her support, not only academically but also professionally and personally. I have endured trials in all phases of my life over the past five years and she was always encouraging and understanding. I would like to thank my committee members, Dr. Cohen, Dr. Stewart, and Dr. Jones, for their guidance, support, and for pushing me to grow academically and professionally throughout the course of my graduate studies.

I would like to thank Dr. Akey, Dr. Swinford, and the many coworkers, colleagues and friends at TVMDL who have supported me in many ways during the course of my graduate studies. I truly appreciate their patience, guidance, and support during this time.

I would like to thank my family: mom and Terry, dad and Dianne, John, Justin, Cameron, Travis, and Cooper for their encouragement during this journey. I am forever grateful for their support and belief in me along the way.

Finally, I would like to thank my wife, Toy, and my two sons, Parker and Caden. Without them, none of this would have been possible. Their unconditional love, encouragement, and patience allowed me to withstand the endurance of this test.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Virginia Fajt (advisor) and Dr. Randolph Stewart from the Department of Veterinary Physiology and Pharmacology, Dr. Noah Cohen from the Department of Large Animal Clinical Sciences, and Dr. Meredyth Jones from the Department of Food Animal Medicine and Surgery at the Oklahoma State University Center for Veterinary Health Sciences.

Felecia Boykin with TVMDL performed the data mining in Chapter II. The studies performed in Chapters III and IV were in collaboration with the College of Veterinary Medicine at North Carolina State University (IACUC #17-132-A), under the direction of Dr. Ronald Baynes. Sample collection and LC-MS analysis in Chapters III and IV were performed in part by Claire Bublitz with the College of Veterinary Medicine at North Carolina State University. The student completed all other work for the dissertation independently.

Funding Sources

Funding for the work conducted in Chapters III and IV was provided by the major livestock shows in the state of Texas.

NOMENCLATURE

ADI	Acceptable Daily Intake
ARCI	Association of Racing Commissioners International
COX	Cyclooxygenase
ELISA	Enzyme-Linked Immunosorbent Assay
FARAD	Food Animal Residue Avoidance Databank
FDA	Food and Drug Administration
FSIS	Food Safety Inspection Services
GFR	Glomerular Filtration Rate
HPLC	High-Performance Liquid Chromatography
IM	Intramuscular
LC-HRAMS	Liquid Chromatography – High-Resolution Accurate Mass Spectrometry
LC-MS	Liquid Chromatography – Mass Spectrometry
LC-MS/MS	Liquid Chromatography – Tandem Mass Spectrometry
LIMS	Laboratory Information Management System
LOD	Limit of Detection
LOQ	Limit of Quantitation
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
NSAID	Nonsteroidal Anti-Inflammatory Drug
PD	Pharmacodynamic
PK	Pharmacokinetic

PO	Per os (oral administration)
TLC	Thin Layer Chromatography
TVMDL	Texas A&M Veterinary Medical Diagnostic Laboratory
USDA	United States Department of Agriculture

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CHAPTER I

INTRODUCTION AND OVERVIEW OF CURRENT REGULATION OF DRUG USE IN LIVESTOCK SHOW ANIMAL SPECIES

Livestock shows utilize drug testing for multiple purposes: to foster fair competition among competitors, to ensure animal welfare, and to protect the food supply. Ante-mortem samples, such as urine, blood, and feces, are screened for a variety of drugs including nonsteroidal anti-inflammatory drugs (NSAIDs), beta-adrenergic agonists with repartitioning effects, anabolic steroids, corticosteroids, stimulants, diuretics, analgesics, sedatives and tranquilizers, antihistamines, local anesthetics, and antibiotics. Drug testing is performed in a variety of show animal species including cattle, pigs, sheep, goats, horses, chickens, turkeys, and rabbits.

Many shows and fairs regulate drug use with a so-called "zero tolerance" policy,[1,2] meaning any substance found in samples from an animal will result in some form of penalty for the exhibitor. "Zero" is defined as the lower limit of detection of the analytical technique employed to test samples. As analytical testing continues to improve and becomes more sensitive, this type of policy may become untenable and indefensible, because very low concentrations of drugs are unlikely to cause physiological effects.

Drugs used in show animals can be divided into 3 categories. The first category includes therapeutic drugs used in a legal manner with no intention of altering performance in the animal. Examples include those approved by the Food and Drug Administration (FDA) to treat, control, or prevent disease, such as antibiotics or anthelmintics. The second category consists of drugs not allowed for use in animals and drugs with no therapeutic effect, used illegally to enhance

performance. Examples include beta-agonists in unapproved species, anabolic steroids, and illicit drugs, such as tetrahydrocannabinol (THC) and methamphetamine. The last category consists of drugs that may be used to treat, control, or prevent disease but are used illegally to enhance performance in animals. For example, flunixin is an NSAID indicated for the control of pyrexia associated with bovine and porcine respiratory diseases, endotoxemia and acute bovine mastitis in cattle,[3] and for reducing fever associated with swine respiratory disease.[4] However, it is also analgesic and at some concentrations can be used to mask lameness.[5-7] Concentrations at which performance-enhancing effects of therapeutic drugs can occur are unknown in species such as cattle, sheep, goats, and pigs. While flunixin is approved for use in cattle and swine, many shows regulate the use of flunixin with a "zero tolerance" policy given its performance-enhancing properties.

One unique aspect of doping in livestock show animals compared to human and equine sports is the added concern of food safety. Most livestock shows test urine as collection is non-invasive, urine allows some drugs to be detected longer than is possible in plasma, and urine allows for the detection of major metabolites for some drugs. However, a challenge facing the livestock show industry is correlating urine or plasma drug concentrations with drug found in tissues at slaughter, also known as residues. The FDA has established tolerances, the maximum concentration of a drug allowed in edible tissues from food-producing animals for human consumption, as well as withdrawal times, the amount of time required before a food-producing animal or product from an animal can be harvested following drug administration. Livestock exhibitors confuse withdrawal time with what is often called "elimination time", or the amount of time required to excrete all of a drug. In fact, the so-called elimination time is longer than the withdrawal time for some drugs. Therefore, following the labeled withdrawal time of a

medication may result in tissue concentrations below the tolerance but still result in detectable drug in urine. As a result, questions arise when positive urine test results are reported. Is it safe for an animal to enter the food supply with a positive urine test? Because the correlation between urine concentrations and tissue residues is mostly unknown, livestock shows choose to regulate some FDA approved drugs with established tolerances and withdrawal times using “zero tolerance”.

Changes in drug testing methods in recent years have improved drug detection limits by as much as 100-fold for some drugs. Improvements in drug testing methods create the need to modify approaches used to regulate drug use in livestock show animal species. While a “zero tolerance” approach is easy to enforce and manage, it lends itself to questions that have yet to be answered. For example, new testing methods allow the detection of therapeutic drugs in urine below 0.5 parts per billion (ppb). Do these trace levels provide performance-enhancing effects in the animal? Another area of concern is environmental exposure to drugs. It is possible for an untreated animal to test positive for a drug from environmental residue contamination.[8,23] An additional issue relates to tissue withdrawal guidelines and detection of drugs in urine. If the labeled withdrawal time is followed, it is possible for a drug to be detected in the urine at the withdrawal time. It is not clear for some drugs how much time must pass before it is no longer detectable in the urine.

Therefore, the purpose of this research was to identify and address data gaps in the current understanding of drug testing and drug disposition, and to integrate those data with published data to propose an approach to standardizing anti-doping policies, particularly those related to therapeutic drug use. Four individual projects comprise this body of work. First, to determine which drugs are most commonly identified in drug testing at livestock shows, a review

of historical drug test results from one laboratory in Texas from 1999 to 2017 was performed. Second, a study was performed to describe the plasma pharmacokinetics and renal clearance of flunixin in goats. While there are currently no NSAIDs approved by the FDA for small ruminants, flunixin is used in sheep and goats as an analgesic. Additionally, urine is the sample of choice when testing for drugs in exhibition animals. There is currently a gap in the scientific literature describing concentrations of flunixin in goat urine. Third, a study was performed to describe the plasma pharmacokinetics and renal clearance of meloxicam in goats. Similar to flunixin, meloxicam is used in small ruminants and little is known about meloxicam concentrations in goat urine. Last, observed and published data, PK/PD modeling results, and measurement uncertainty were integrated to propose a method for establishing regulatory thresholds and decision limits for therapeutic drugs detected in urine from animals exhibited at livestock shows.

CHAPTER II

ANALYSIS OF REPORTED DRUG TEST RESULTS FROM LIVESTOCK SHOW ANIMALS FROM TEXAS AND OTHER STATES IN THE UNITED STATES (1999 – 2017)

Introduction

Doping, the use of drugs to enhance performance, is a familiar term as it applies to human and equine athletes. The modern era of doping dates to the early 1900s, with the illegal use of performance-enhancing chemicals in racehorses. Its use in the Olympics was first reported in 1904.[9] Doping also occurs in food animal species, such as cattle, sheep, goats and pigs that are exhibited at livestock shows and county fairs. Media attention garnered in the show animal industry is significantly less than that associated with human and equine sports because most people do not associate the term doping with livestock show animals.

Every year, thousands of youth across the United States (US) invest hours of time and energy working with animals such as cattle, sheep, goats, and pigs, preparing them for livestock shows and county fairs. These youth then exhibit the animals for judging at the show on phenotypical traits such as muscling, structural correctness, frame-size, style, and balance. Awards, including trophies, ribbons, belt buckles, and scholarship money, are earned by exhibitors whose animals place at the top of their respective class. The size of the show varies from local county fairs to state and national exhibitions. Regardless of size, the level of competitiveness is high at every show.

An auction often follows the show where the animals are purchased by local businesses and individuals who desire to contribute to the scholarship funds for the youth. In some cases, animals can generate thousands of dollars in prize and scholarship money. For example, the

highest amount ever paid for a show animal was in 2001 at the Houston Livestock Show & Rodeo where the Grand Champion Steer was purchased at auction for $600,000.[10] The level of competition is such that some parents and exhibitors feel compelled to perform illegal acts to gain an advantage over other competitors. Similar to human and equine doping, one of the more common methods is the use of drugs to enhance the performance of the animal. Unlike human and equine doping, doping in livestock show animal species creates a unique risk to food safety, because many of these animals end up in the food supply.

Anti-doping regulation involves testing athletes to detect prohibited drug use. Drug testing in racehorses began by testing saliva in 1934.[11] The first reported drug testing of human athletes occurred in 1966.[9] Similarly, livestock shows and county fairs across the country implement drug testing programs to create fairness among competitors, protect animals, and ensure safety of the food supply chain. The Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL) has been providing drug testing services for county fairs and livestock shows across the country since 1989.

It is important to mention that post-mortem tissue samples are subject to drug testing by federal regulation at the time of harvest to ensure food safety. However, comparative post-mortem data are not available from the animals tested by TVMDL from 1999 to 2017. Ante-mortem samples, such as urine, blood, and feces, are collected at the show and immediately submitted to a testing laboratory. The testing laboratory screens the samples for drugs and drug metabolites by a variety of methods that include thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and gas chromatography coupled with mass spectrometry (GC-MS). Testing laboratories screen for a variety of drugs including NSAIDs, beta-adrenergic

agonists with repartitioning effects, anabolic steroids, corticosteroids, stimulants, diuretics, analgesics, sedatives and tranquilizers, antihistamines, local anesthetics, and antibiotics. Drug testing is performed in a variety of show animal species including cattle, pigs, sheep, goats, horses, chickens, turkeys, and rabbits.

The drug testing laboratory at TVMDL, certified by the International Organization for Standardization (ISO 17025), provides drug testing services for the livestock and poultry industries, and the horse and greyhound racing industries. TVMDL has been providing drug testing for the livestock show industry for approximately 27 years. Our laboratory provides drug testing for more than 90 shows and county fairs across 16 different states. Electronic test results for the last 18 years were reviewed.

An objective of this retrospective study was to present descriptive statistics. To the authors' knowledge, this is the first publication of this type data.

Our second objective was to look for trends and to provide information to educate stakeholders, youth, and leaders within the livestock show industry. Changes in testing technology and analytical capabilities in recent years have led to detection of drugs in urine at much lower concentrations than ever before. It is important to share this information with livestock show and fair boards to allow modification of policies and procedures, and adjust to improvements in testing.

Information from this study would also inform exhibitors, parents, county agents, agricultural science teachers, and veterinarians. Educating these stakeholders about testing capabilities and test results can make them aware of the ability of test methods to detect trace amounts of drugs. Using proper withdrawal times, avoiding use of illegal drugs, and

understanding the potential for an animal to be exposed to trace levels of drugs from the environment are all important approaches for reducing positive test results.

Our third objective was to identify areas for potential research opportunities. Enhanced testing capabilities create need for better understanding of the significance of detectable drug presence in urine. Questions arise regarding application and relevance of low concentrations of drugs in urine, how they correlate with enhanced performance, and how they correlate with tissue levels to ensure food safety. Currently, a gap exists in the understanding of presence of a chemical substance and the likelihood that it provides an enhanced performance or physiologic effect. This drug effect or pharmacodynamics has the potential to lead to unfair competition at livestock shows, so correlation of detectable drug concentration with effects in animals is necessary. Understanding which drugs are more frequently identified in drug testing will establish priorities for future efforts.

Materials & Methods

Drug testing data from 1999 to 2017 were queried from 2 different laboratory information management systems (LIMS) using Microsoft SQL Server Management Studio (Redmond, WA). Microsoft Access was used to format the data into a single style. Pre-determined search criteria were utilized to filter and collect data. These criteria included test result (Positive/Negative), drug identified, animal species, sample matrix (*e.g.*, urine, feces or retina tissue), and year. Basic statistical analyses were performed using Microsoft Excel. Identification of specific livestock shows was not included in order to maintain client confidentiality and anonymity.

Methods used to perform drug testing included thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography

(HPLC), liquid chromatography – tandem mass spectrometry (LC-MS/MS), and Orbitrap™ liquid chromatography – high-resolution accurate mass spectrometry (LC-HRAMS). Screening methods included TLC, ELISA and LC-HRAMS, while confirmation methods included HPLC and LC-MS/MS (Table 1).

Year	Screening Method	Confirmation Method
1999	TLC	HPLC
2000	TLC	HPLC
2001	TLC	HPLC
2002	TLC and ELISA	HPLC
2003	TLC and ELISA	HPLC
2004	TLC and ELISA	HPLC
2005	TLC and ELISA	HPLC
2006	TLC and ELISA	HPLC
2007	TLC and ELISA	LC-MS/MS
2008	TLC and ELISA	LC-MS/MS
2009	TLC and ELISA	LC-MS/MS
2010	TLC and ELISA	LC-MS/MS
2011	TLC and ELISA	LC-MS/MS
2012	TLC and ELISA	LC-MS/MS
2013	TLC and ELISA	LC-MS/MS
2014	TLC and ELISA	LC-MS/MS
2015	LC-HRAMS	LC-MS/MS
2016	LC-HRAMS	LC-MS/MS
2017	LC-HRAMS	LC-MS/MS

Table 1 Methods used for screening and confirmation testing (1999 to 2017). [Thin Layer Chromatography (TLC), Enzyme-Linked Immunosorbent Assay (ELISA), High-Performance Liquid Chromatography (HPLC), Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS), Liquid Chromatography – High-Resolution Accurate Mass Spectrometry (LC-HRAMS)]

Results

During 1999 to 2017, TVMDL provided drug testing for 11 to 88 shows (Figure 1). In 2017, that included 57 from Texas and 32 outside of Texas (Figure 1).

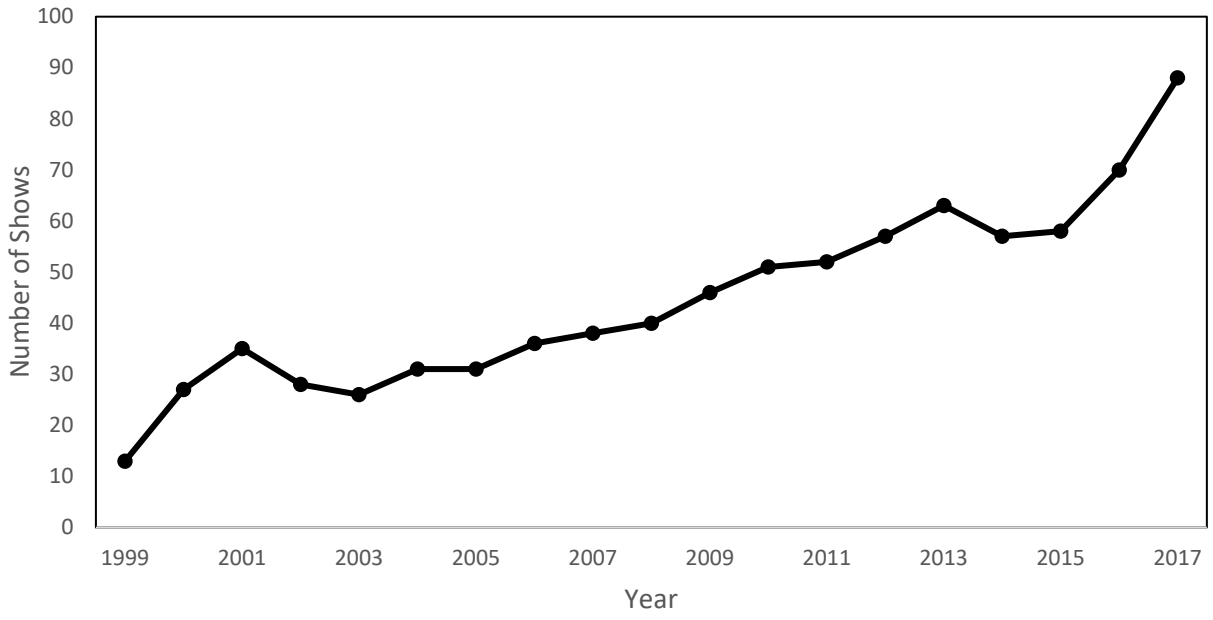

Figure 1 Number of livestock shows tested annually by the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL) (1999 to 2017).

Various animal matrices were submitted for drug testing in show animals (Table 2). Urine was the most common sample submitted. Fecal samples were submitted from chickens, turkeys, and rabbits. Retinal samples were submitted from cattle, pigs, sheep, and goats to detect beta-adrenergic agonist drugs with repartitioning effects. Liver, kidney, and muscle samples were submitted from cattle, sheep, goats, pigs, chickens, and turkeys. Contraband samples included swabs and gauze pads taken from different anatomical locations to detect the presence of topical medications. Samples labeled as “suspect” included those determined to be potentially

positive following the initial screen, but the volume of sample remaining was insufficient for confirmation testing.

Sample Matrix	Total Tested	Positive	Suspect
Urine	29,238	1,525 (5.2%)	5
Feces	1,094	44 (4.0%)	0
Serum	842	46 (5.5%)	0
Retina	803	57 (7.1%)	0
Tissue (Liver, Kidney, Muscle)	50	2 (4.0%)	0
Contraband	8	8 (100%)	0

Table 2 Number of each type of sample tested, including number positive, number negative, and number of suspect samples (1999 to 2017).

A total of 26,516 urine samples, 1,395 of which were positive (5.3%), were tested from cattle, sheep, goats and pigs (Figure 2): 394 of 8,149 (4.8%) cattle tested positive; 738 of 6,409 (11.5%) swine tested positive; 116 of 5,211 (2.2%) goats tested positive; and, 145 of 6,621 (2.2%) sheep tested positive. The highest annual number of positive samples from the combination of cattle, pigs, sheep, and goats was reported in 2015, during which 331 of 1,917 (17.3%) tested positive. In 2015, 195 of 466 (41.8%) swine samples were reported positive, including 97 for ractopamine, 20 for caffeine, 19 for flunixin, 16 for theobromine, and 10 for theophylline. The total number of positive urine samples peaked in 2015, followed by a decline in 2016 and 2017 to 280 and 124 positive urine samples, respectively.

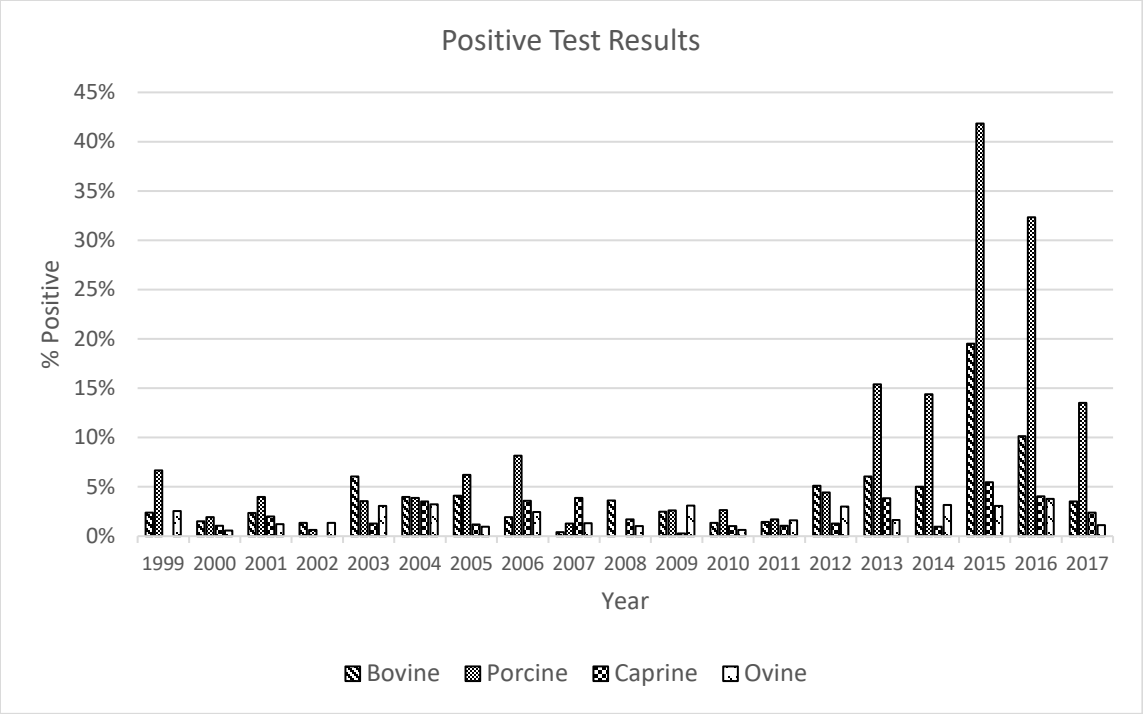

Figure 2 Percent positive urine test results by species (1999 to 2017).

Fifteen (15) of 26,516 (0.06%) urine samples from cattle, sheep, goats and pigs tested positive for multiple drugs. Three bovine samples tested positive for both ractopamine and zilpaterol. One caprine sample tested positive for flunixin, lidocaine, hydroxylidocaine, clenbuterol, and dexamethasone. A second caprine sample tested positive for flunixin and ractopamine. One ovine tested positive for flunixin and methylprednisolone. Four swine samples tested positive for flunixin and dexamethasone. One swine sample tested positive for sulfamethazine and acetylsalicylic acid. One swine sample tested positive for flunixin and sulfamethazine. One swine sample tested positive for flunixin and methylprednisolone. One swine sample tested positive for dexamethasone and naproxen. One swine sample tested positive for flunixin, ractopamine and zilpaterol.

Generally, TVMDL performs qualitative testing on samples from show animals. Occasionally, shows and fairs request a quantitative test to assess the concentration of a therapeutic drug detected in urine. While threshold levels of therapeutic drugs have not been established in show animal species, a quantitative test is available to determine if the concentration of a therapeutic drug detected in urine was low or high. From 2016 to 2017, TVMDL performed a quantitative test on 25 animals that tested positive for flunixin (see Figure 3). The concentration of flunixin ranged from 0.6 to 3,600 ng/mL.

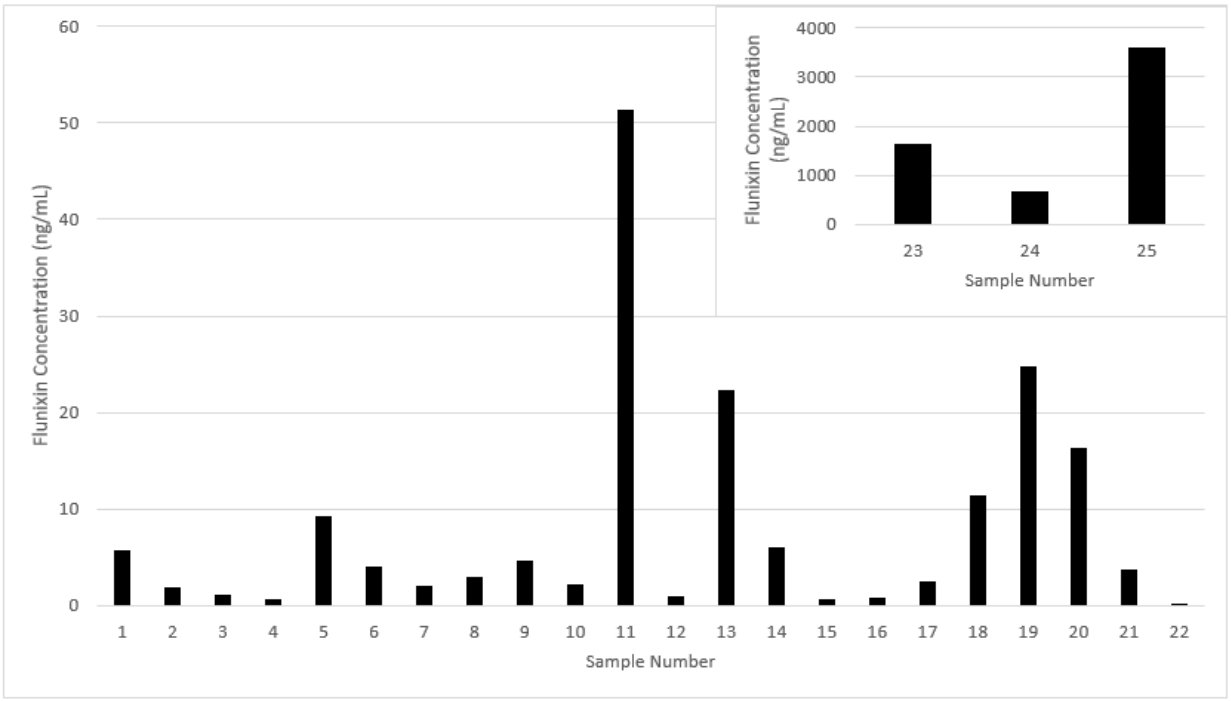

Figure 3 Range of quantified urine concentrations of flunixin (2016 to 2017). (Reported as "Positive" in bovine, porcine, ovine, and caprine samples) (n = 25)) [inset = outliers with very high concentrations]

A total of 42 different drugs and metabolites were confirmed in urine samples from 1999 to 2017 (Table 3). Combined, ractopamine and flunixin accounted for almost half (48.7%) of all positive findings in urine.

Drug	Number Confirmed (1,451 Total)
Ractopamine	490 (33.8%)
Flunixin	252 (17.4%)
Sulfamethazine	128 (8.8%)
Zilpaterol	86 (5.9%)
Dexamethasone	74 (5.1%)
Theobromine	74 (5.1%)
Caffeine	70 (4.8%)
Lidocaine + Metabolites	49 (3.4%)
Phenylbutazone + Metabolites	48 (3.3%)
Furosemide	37 (2.6%)
Methylprednisolone	31 (2.1%)
Theophylline	24 (1.7%)
Nicotine + Metabolites	24 (1.7%)
Sulfadimethoxine	21 (1.5%)
Acetylsalicylic acid	17 (1.2%)
Sulfadiazine	14 (1.0%)
Procaine	11 (0.8%)
Firocoxib	10 (0.7%)
Paraxanthine	9 (0.6%)
Trimethoprim	8 (0.6%)
Ibuprofen	7 (0.5%)
Acepromazine + Metabolites	6 (0.4%)
Isoflupredone	6 (0.4%)
Chlorpromazine + Metabolites	5 (0.3%)
Meloxicam	5 (0.3%)
Chlortetracycline	5 (0.3%)
Sulfamethoxazole	5 (0.3%)
Clenbuterol	5 (0.3%)
Boldenone	4 (0.3%)
Naproxen	3 (0.2%)
Diphenhydramine	3 (0.2%)
Aminoantipyrine + Metabolites	3 (0.2%)
Azaperone + Metabolites	2 (0.1%)

Dextrorphan	2 (0.1%)
Altrenogest	2 (0.1%)
Mepivacaine	2 (0.1%)
Albuterol	2 (0.1%)
Xylazine	2 (0.1%)
Terbutaline	1 (0.07%)
Diclofenac	1 (0.07%)
Ketoprofen	1 (0.07%)
Meclofenamic Acid	1 (0.07%)

Table 3 Drugs confirmed in urine and number of animals confirmed positive for each drug (all species) (1999 to 2017).

A total of 1,094 fecal samples were tested from 1999 to 2017 (Table 4). Ractopamine made up the majority of positive samples (88.6%; 39/44 positives) and 3.6% of total samples.

Drug	Positive
Ractopamine	39
Flunixin	1
Tramadol	1
Procaine	1
Lidocaine	1
Testosterone	1
Total Tested	1,094

Table 4 Drugs confirmed in feces samples from poultry species (1999 to 2017).

Three beta-adrenergic agonist drugs, clenbuterol, zilpaterol, and ractopamine, were screened in 803 retinal samples (Table 5). Positive samples were found in 33 of 454 (7.3%) sheep and 9 of 267 (3.4%) goats. Ractopamine is approved by the FDA for finishing swine such that many shows do not consider it a banned substance in this species. The animal species was not provided to TVMDL for 49 of the animals, of which 3 tested positive for ractopamine.

	Bovine	Porcine	Ovine	Caprine	Unknown
Clenbuterol	0	0	10 (2%)	2 (0.7%)	0
Zilpaterol	2 (9%)	0	23 (5%)	5 (2%)	0
Ractopamine	0	9 (90%)	0	2 (0.7%)	3 (6%)
Total Tested	23	10	454	267	49

Table 5 Drugs confirmed in retina samples from bovine, porcine, ovine, and caprine species (Total Number and % Positive) (1999 to 2017).

Discussion

The data obtained during the last 19 years offers a unique look at livestock shows. While food safety is of highest importance, testing ante-mortem samples, such as urine, offers more insight into drugs used to gain a competitive edge over competition and potentially enhance the performance of the animal. The majority of the drugs that were confirmed in urine from cattle, sheep, goats, and pigs had the potential to enhance performance in the animal. TVMDL tested urine samples for antibiotics (sulfamethazine, sulfadiazine, sulfamethoxazole, sulfadimethoxine, trimethoprim, and chlortetracycline) until 2016. It is difficult to correlate detection of most antibiotics in urine with potential violative tissue residues, and antibiotics are not considered

performance-enhancing drugs. Therefore, TVMDL no longer tests for antibiotics in ante-mortem samples from show animals.

Test methods used to screen livestock show samples between 1999 to 2014 included TLC and ELISA. Test methods used to perform confirmation testing included HPLC (1999 to 2006) and LC-MS/MS (2007 to 2017). In 2015, the screening method changed to LC-HRAMS. As a result, the capability to detect drugs at lower concentrations improved significantly. This coincides with a steep increase in the number of positive urine samples in cattle and pigs from 2014 to 2015 (see Figure 2). While increased drug administration to these animals may have contributed to the rise in the number of positive findings, it was more likely due to the improved sensitivity of the new screening method.

Many shows and fairs regulate drug use with a so-called "zero tolerance" policy,[1,2] meaning no drug is allowed in the animal at the time of the show at any concentration. "Zero" is defined as the lower limit of detection of the analytical technique employed to test samples. As analytical testing continues to improve and becomes more sensitive, this type of policy may need to be reconsidered. In order to address this issue, some shows conduct investigations to determine the circumstances that led to a positive test result. Exhibitors found in violation are often times offered an opportunity to explain the details of their case in a formal appeals hearing. Penalties can range from withholding premiums to a lifetime ban, depending on the drug that was used and the circumstances surrounding the use.[2]

Regulation of drug use in show animals can be divided into 3 categories. The first category includes therapeutic drugs used in a legal manner with no intention of altering performance in the animal. Examples include those approved by the FDA to treat, control, or prevent disease. The second category consists of drugs not allowed for use in animals and drugs

with no therapeutic effect used illegally to enhance performance. Examples include beta-agonists in unapproved species, anabolic steroids, and illicit drugs, such as tetrahydrocannabinol (THC) and methamphetamine. The last category consists of therapeutic drugs used illegally to enhance performance in the animal. For example, flunixin is indicated for the control of pyrexia associated with bovine and porcine respiratory diseases, endotoxemia, and acute bovine mastitis in cattle,[3] and for reducing fever associated with swine respiratory disease.[4] However, it is also considered an analgesic and at certain concentrations can be used to mask lameness.[5-7] Concentrations at which performance-enhancing effects of some therapeutic drugs occur can be difficult to measure in species such as cattle, sheep, goats, and pigs. While flunixin is approved for use in cattle and swine, many shows regulate the use of flunixin with a "zero tolerance" policy given its performance-enhancing properties. Pharmacokinetic studies are underway to evaluate this policy, and to provide livestock show and fair boards the scientific data required to adjust or establish rules and policies regarding the use of therapeutic medications in show animals.

One unique aspect of doping in show animals compared to human and equine sports is the added risk to food safety. A challenge facing the livestock show industry is correlating urine and plasma drug concentrations with residual tissue concentrations. The FDA has established tolerances, the maximum concentration of a drug allowed in edible tissues from food-producing animals for human consumption, as well as withdrawal times, the amount of time required before a food-producing animal can be harvested following drug administration. Many stakeholders in the livestock show industry confuse withdrawal time with elimination time (*i.e.*, the amount of time required to excrete all of a drug). In fact, the elimination time can be longer than the withdrawal time for some drugs and is highly dependent upon the sensitivity of the analytical

method used to detect the drug. Therefore, following the labeled withdrawal time of a medication will ensure tissue concentrations are below the tolerance however, it is still possible to generate a positive urine test. Unfortunately, the correlation between urine concentrations and tissue residue concentrations is generally unknown. As a result, certain FDA approved drugs with established tolerances and withdrawal times are regulated by "zero tolerance". The improved sensitivity gained by changing the screening method in 2015 led to an increase in the number of confirmed positive therapeutic drugs, like flunixin, especially at lower concentrations (see Figure 3). Some of these low concentrations were likely the result of uncertainty in knowing how to modify therapeutic regimens and withdrawal times. Current research efforts are in progress to better understand these low concentrations, such as assessing renal clearance and elimination time, correlating urine concentrations with tissue concentrations for food safety, and establishing conservative urine withdrawal times. Hopefully, results of these studies will enable interpretation of laboratory results in terms of both clinical use and food safety.

It is also important to consider the increase in the number of positive findings in urine attributable to environmental substances. While caffeine and theobromine are considered environmental contaminants, they are stimulants that can cause changes in an animal's performance and behavior, and are thus prohibited in show animals. Their psychostimulant properties cause psychomotor-activating, reinforcing, and arousing effects[12] that can cause stubborn and lethargic animals to appear alert and responsive. Caffeine is also a diuretic. Diuretics increase the excretion of water from the body thereby accentuating muscle definition and body tone.[13] Diuretics can act as masking agents by hastening drug removal from the body with increased urine production.[13] As with therapeutic drugs, concentrations at which performance-enhancing effects can occur from drugs like caffeine and theobromine in species

such as cattle and pigs are unknown. The improved sensitivity due to test method changes in 2015 also led to a significant increase in the detection of caffeine and theobromine in urine, especially at lower concentrations. The number of caffeine positive urine samples from 2014 to 2015 increased from 3 to 29 in cattle and pigs combined. The number of theobromine positive urine samples from 2014 to 2015 increased from 2 to 21 in cattle and pigs combined. While it is not possible to differentiate environmental exposure from intentional administration, the improved sensitivity of the screening method exposed the possibility of environmental contamination from these drugs.

Testing retinal tissue provides an effective way to detect forbidden drug presence. Certain drugs like zilpaterol, clenbuterol, and ractopamine can remain in retina tissue for several months. These beta-adrenergic agonist drugs are considered repartitioning agents for their ability to increase feed conversion efficiency and carcass lean content. Illegal use of these drugs, particularly in sheep and goats, often occurs in the months leading up to competition. Carefully scheduled withdrawal of these drugs in advance of the show or fair increases difficulty detecting their presence in urine. A considerably higher percentage of sheep and goat retinal samples tested positive for either clenbuterol, ractopamine, or zilpaterol, compared to only 0.7% of the sheep and goat urine samples.

Fecal data were obtained from chickens, turkeys, and rabbits. Ractopamine was confirmed in 39 poultry animals. Topmax™ is a product that contains ractopamine hydrochloride and is FDA-approved for use in turkeys to improve weight gain and feed efficiency in finishing birds. In most cases, the distinction between chicken and turkey was not made by the show when the samples were submitted to TVMDL. Therefore, it is not possible to determine how many of the 39 positive findings were in an approved (turkey) versus unapproved (chicken) species. In

the US, it is illegal to administer drugs extralabel in feed. Thus, it is important to identify the species when making a determination about penalties for a competitor with a positive sample.

While violative test results garner the most attention, it is important to highlight the importance of the 95% of urine samples that tested negative. A positive test result can be damaging to a show's reputation or the industry as a whole. The high percentage of negative test results from our laboratory from 1999 to 2017 is good news. As a whole, the majority of stakeholders involved in raising and exhibiting show animals did not participate in the illegal administration of performance-enhancing drugs.

Future Directions

People enticed to cheat to gain an advantage over their competitors will employ new methods and strategies to try to avoid detection. Therefore, studies on alternative test methods and sample types are needed to complement current methods to detect illegal drug use. For example, hair testing is increasingly used in other performance arenas, such as horseracing.[14] Researchers have demonstrated the ability to detect anabolic steroids and their esters in horse hair.[15] Hair testing is also used in people to detect drugs of abuse[16] and to evaluate recidivism before an offender of impaired driving can regain his or her driver's license.[17] The ability to detect illegal drug use retrospectively using hair has the ability to change the landscape of drug testing as a whole. Another area gaining traction in doping testing is using biomarkers indicative of illegal drug use, rather than testing for the actual drug.[18] A biomarker refers to a quantifiable biological parameter that is measured and evaluated as an indicator of normal biological, pathogenic, or pharmacologic responses to a therapeutic intervention, as defined by the National Institutes of Health.[19] Biomarkers are currently used by the US Anti-Doping Agency (USADA) to create an Athlete Biological Passport (ABP). An ABP is the monitoring of selected biological

parameters over time that may indirectly reveal effects of doping on the body. This approach allows anti-doping organizations to generate individual, longitudinal profiles for each athlete and to look for any fluctuations that may indicate the use of performance-enhancing drugs or methods.[20] Biological passports are also being explored in the equine industry.[21,22]

Conclusions

Individual animal drug test results are considered sensitive and confidential information. However, categorizing and reviewing the summation of test results over time provides a unique opportunity to make observations and identify trends. These data can be used to identify and prioritize drugs in need of further investigation and subsequent studies to provide a better understanding of the disposition of these drugs in food animal species. As testing platforms improve, it is important to understand how the evolution of drug testing may affect the regulation of drugs used in show animal species. The concentrations of flunixin quantified during 2016 to 2017 offer an example. While some of these concentrations were quite low, it is unknown if they resulted in performance-enhancing effects.

Of the 26,390 urine samples tested between 1999 to 2017 from cattle, sheep, goats, and pigs, 1,393 were confirmed positive (5.3%) The annual percent positive samples from these 4 species combined ranged from 1.1% to 17.3% during this period. The drug testing program at TVMDL has evolved over the last 19 years, with the most dramatic changes made in the last 3 years. Initially, the number of drugs tested in show animals was quite small. This number has grown substantially, although there are likely other drugs used in show animals that we are unable to detect. The occurrence of positive test samples throughout these years demonstrates a continued need for drug testing programs in livestock show animals.

CHAPTER III

PLASMA PHARMACOKINETICS AND RENAL CLEARANCE OF FLUNIXIN IN GOATS*

Introduction

Flunixin meglumine (*e.g.*, Banamine®) is a nonsteroidal anti-inflammatory drug (NSAID) indicated for alleviation of inflammation and analgesia. For example, it is used to control pyrexia associated with bovine and porcine respiratory diseases, endotoxemia and acute bovine mastitis in cattle,[3] and for reducing fever associated with swine respiratory disease.[4] Flunixin works by blocking cyclooxygenase (COX), the enzyme responsible for the direct synthesis of prostaglandins, which are involved in inflammatory processes through their role in vasodilation, fever and pain.

Flunixin is commonly used in food animals, and it is one of the more commonly detected drugs in exhibition animals (Table 3). While flunixin is indicated for therapeutic use, it can also be used to alter the performance of an animal by masking pain during competition. Regulation of therapeutic drugs, like flunixin, used in exhibition animals is complicated. Improvements made to drug testing methods in recent years make it possible for an exhibition animal treated in a legal manner with an approved therapeutic drug prior to competition to test positive. Low level exposure to some drugs can occur from environmental[8,23] or feed contamination.[24] Increased sensitivity achieved by current analytical testing platforms allows the detection of physiologically unimportant plasma or urine concentrations of therapeutic drugs for extended periods (days or weeks) after their administration.[25] Because of the ability to detect very low,

*Reprint of published data from Small Rumin. Res. 2019. 174:40-46.

insignificant concentrations of therapeutic drugs, a “zero tolerance” approach for regulation of therapeutic drugs, like flunixin, may be too stringent. Establishing decision limits that differentiate between physiologically important and unimportant concentrations of therapeutic drugs may be more appropriate. However, the performance-enhancing effects of NSAIDs at very low concentrations are unknown.

In addition to the use of drug testing to detect unfair practices, regulation of therapeutic drugs used in exhibition animals is important for food safety. Once a livestock project is completed, the animal will enter the food supply. Exhibition animals that enter the food supply represent a large number of carcasses across the country. In 2004 in Texas alone, it is estimated that more than 14 million pounds of carcasses from livestock projects entered the food supply.[26] While the Food Safety Inspection Service (FSIS) tests tissues from carcasses for illegal drug residues, urine is often collected at livestock show exhibitions and tested for performance-altering substances. Urine concentrations of flunixin have been evaluated in camels,[27] pigs,[28] horses,[29] dogs,[30] and cattle.[31] However, little is known about flunixin concentrations detected in urine from goats, and how these levels correlate to plasma and tissue concentrations.

Pharmacokinetic (PK) studies are performed to evaluate the movement of a drug into, throughout and out of the body, that is, the time course of its absorption, bioavailability, distribution, metabolism, and excretion.[32] These studies usually involve the collection of multiple plasma, urine, and in some cases tissue, samples over time. There are currently no published PK studies for evaluating the correlation between plasma, urine, and tissue concentrations of flunixin in goats.

The legitimate use of therapeutic drugs in some animals species, like sheep and goats, presents a third challenge for regulating therapeutic drugs in exhibition animals. Veterinarians

face challenges when treating small ruminants, especially those exhibited at competitions, as there are currently no NSAIDs approved by the Food and Drug Administration (FDA) for small ruminants. Flunixin can be used extralabel only if prescribed by a licensed veterinarian in the context of a valid veterinarian-client-patient relationship and all other requirements of the Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1994.[33] Additionally, a tolerance for flunixin in goats does not exist. Thus, detection of any amount of flunixin in edible tissues from goats at harvest will result in a violation of federal law. This in turn can make it difficult for practitioners to provide guidance on meat withdrawal times to their clients. A meat withdrawal time for flunixin has been established for cattle and is based on an approved tolerance; however, veterinarians cannot use this information to estimate a withdrawal time to use in sheep and goats to achieve no detectable levels of flunixin in meat.

PK studies can provide estimates of pharmacokinetic parameters, such as elimination half-life, that could allow regulators to modify rules about drug violations as drug testing platforms change. These studies can also aid veterinarians by enabling them to provide guidance to their livestock show clients and avoid jeopardizing their ability to exhibit an animal at the expense of the animal's health. The objectives of this study were to describe plasma and urine PK including renal clearance of flunixin administered intramuscular in goats, to evaluate the effectiveness of plasma as a viable sample for drug testing in exhibition animals, and to estimate a conservative urine withdrawal time.

Materials and Methods

Animals and Housing

Five (5) castrated male Boer goats, between 5 to 8 months old and weighing 22.2 to 36.4 kg at the time of dosing, were used for this study. Each animal was housed in individual

metabolism cages. The metabolism cage dimensions (length by width by height) were as follows: 5.5 x 2.5 x 3.5 feet and were specifically designed for small ruminant metabolism research. The cages were cleaned prior to the study and configured with the correct sizing of the head gates to allow the goats free access to water, feed, and hay. Each cage was separated by a minimum of 3 feet. The goats were placed in the metabolism cages approximately 24 hours prior to dosing to allow them to acclimate. The goats were fed a commercial goat feed (Purina Mills) twice daily with free access to water and coastal Bermuda grass hay *ad libitum* throughout the study. Each goat received a physical examination prior to the study, and clinical examinations of all body systems were unremarkable, although liver and kidney function tests were not performed prior to the start of the study. Given that these organs are important for drug clearance, any underlying variability in organ function may be reflected in variability in pharmacokinetic parameter estimates from this study. None of the goats had any previous history of disease or prior exposure to NSAIDs before beginning the study.

Drug Administration and Blood Collection

The goats were weighed on a digital scale the morning of study commencement to determine the appropriate administration dose. Approximately 24 hours prior to dosing, the goats were restrained and sedated for intravenous catheter placement. The area where the catheter was inserted was clipped and scrubbed with alternating swabs of chlorhexidine and isopropyl alcohol. Using sterile technique, a 16 G x 3.25 in. (Angiocath™, BD, Franklin Lakes, NJ, USA) was inserted into the right jugular vein with an extension set and sutured to the skin using 2-0 monofilament suture. Catheters were flushed twice daily with 6 mL of 10 units/mL heparin saline. A single intramuscular dose of flunixin meglumine (2.2 mg/kg; Flunixiject, 50 mg/mL, Henry Schein Animal Health, Dublin, OH) was administered in the lateral aspect of the cervical

neck on the opposite side of the jugular catheter. Injection sites were monitored daily for swelling. Following intramuscular administration, all goats vocalized during injection, but no swelling was noted at the injection site for the duration of the study. Flunixin was administered intramuscular as this route is anecdotally more commonly used in goats than the labeled intravenous route in cattle. Blood samples were taken from the jugular vein and were transferred to lithium heparinized tubes at 0 (pretreatment), 0.25, 0.5, 1, 2, 6, 10, 18, 36, 60, 84, 96, and 120 hours post administration of flunixin meglumine.[34] The samples were stored on ice until centrifugation. The samples were centrifuged at approximately 4,500 g for 10 minutes to yield plasma. The plasma samples were then stored at -80°C until analysis.

Urine Collection

Urine was collected by placing stainless steel buckets beneath the floor of the metabolism cages. A wire mesh was placed across the top of the buckets to prevent feces contamination of the urine. Urine was collected at 0 (pretreatment), 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 336, and 360 hours following drug administration.[34] The total volume of urine produced was measured and recorded at each time point. The buckets were cleaned between each sampling to prevent contamination and sample dilution. The pH of the urine was also measured and recorded at each time point using pH paper (Hydrion, Micro Essential Lab, Inc. Brooklyn, New York). Each urine sample represents fluid collection over time (*e.g.*, not an instantaneous sample) therefore, the total amount of drug excreted at each time point was calculated using the total urine volume measured for each sample. Urine samples were stored at -80°C until analysis.

Postmortem Tissue Collection

Each goat was humanely euthanized after 360 hours following sedation with xylazine using an intravenous dose of 1 mL/10 lbs. of pentobarbital sodium and phenytoin sodium. Approximately 10 grams of liver were collected from the caudal lobe on the right side of each goat.[34]

Drug Analysis

Plasma and urine samples were analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) following protein precipitation.[34] A reference standard for flunixin meglumine (purity >99%) was purchased from Sigma Aldrich. Corrections were made for the meglumine salt in flunixin prior to preparation of standard solutions. The LC-MS/MS method was validated using blank plasma and urine from goats not enrolled in the study. Plasma samples were thawed and extracted using a previously described method.[35] Briefly, 100 µL of each sample was combined with 500 µL of 0.5% citric acid in acetonitrile. Samples were vortexed for approximately 10 seconds and then centrifuged at 4500 RPM for 5 minutes. The supernatant from each sample was transferred into a clean glass borosilicate (16 mm x100 mm) culture tube. The supernatant was then dried via Rapid Vac Evaporation at 55°C under a stream of nitrogen (24 psi) for approximately 15 minutes. Each sample was reconstituted in 300 µL of 50:50 acetonitrile:deionized water (ddH_2O) with 0.1% formic acid, and vortexed for approximately 30 seconds. The samples were then filtered through a 0.2 µ PTFE Whatman Mini-uniprep syringe filter and analyzed by LC-MS/MS. The injection volume was 5 µL for all samples. The mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile at a flow rate of 0.4 mL/min. A gradient was applied beginning at 30% B maintained for 1 minute, increasing to 90% B in 1 minute and maintained at 90% B for 1.5 minutes, followed by re-

equilibration to 30% B for 1.5 minutes. Chromatographic separation was achieved using a BEH Phenyl 1.7 µm 2.1 x 100 mm column (Waters, Milford, MA) and maintained at 40°C. Flunixin eluted at 2.4 minutes. Calibration standards were prepared over a linear range for each sample matrix. The calibration solution ranged from 0.5 to 500 ng/mL for plasma and urine. The LOQ and LOD were determined to be 5 ng/mL and 0.5 ng/mL, respectively. The run time was 5 minutes for each injection.

PK Analysis

Drug concentrations were analyzed using commercially available software (Phoenix® WinNonlin® version 6.3; Certara USA, Inc., Princeton, NJ, USA) to determine PK parameters for each goat. Noncompartmental analysis (weighted $1/Y^2$) was used to determine the terminal rate constant (λ_z), half-life of the terminal phase ($t_{1/2\lambda}$), and total area under the curve (AUC) for flunixin. The maximum concentrations (C_{max}) and time to maximum concentration (T_{max}) in plasma and urine were also determined for each goat. Individual renal clearance values, corrected for body weight, were estimated for each goat using the following equation[36]:

$$Renal\ Clearance\ \left(\frac{mL}{hr}\right) = \left(\frac{Ae}{AUC} * BW\right);$$

where Ae is the cumulative amount of drug excreted unchanged in the urine up to 360 hours, AUC is the area under the plasma drug concentration versus time curve to infinity, and BW is the body weight (kg) of each individual goat.

Results

The original data was collaboratively obtained and shared between two different institutions. Manipulation of the data was performed independently. One form of the data has been reported.[34]

Flunixin in Plasma

Median plasma flunixin concentrations over time following a single intramuscular injection of 2.2 mg/kg are presented in Figure 4.

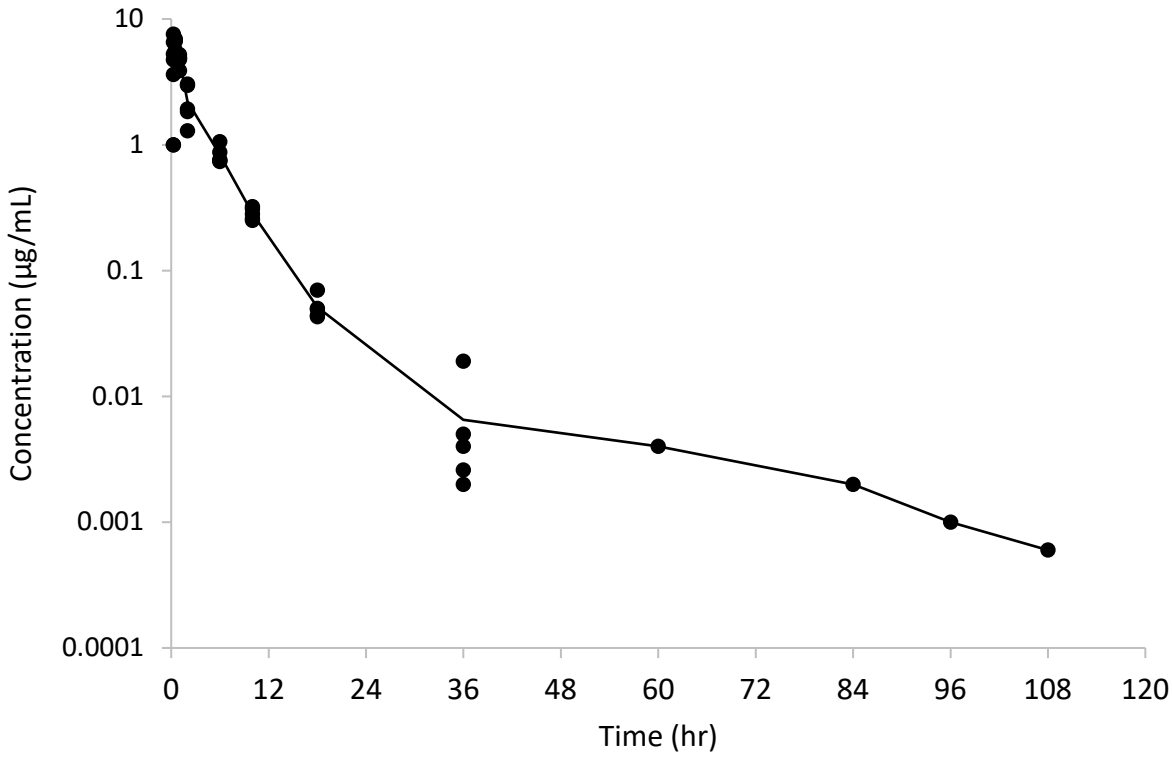

Figure 4 Plasma flunixin concentration versus time profile from 5 goats after a single intramuscular dose of flunixin meglumine (2.2 mg/kg). Note: Plasma data are expressed as

individual points; the solid line represents the mean of all goats. [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

Plasma PK variables for each goat that describe the disposition of flunixin following a single intramuscular injection are presented in Table 6. Flunixin concentrations in plasma were below the limit of quantitation (LOQ) (5 ng/mL) in 4 of 5 goats at 36 hours post drug administration. Plasma flunixin concentrations were below the LOQ in the 5th goat at 60 hours following drug administration. The median volume of distribution (Vd) (908 [627.9-2631] mL/kg) appears to be large however, data from intravenous administration are needed to assess the significance of this value.

PK Parameter	Goat 1	Goat 2	Goat 3	Goat 4	Goat 5	Mean	SD	Median
r^2	0.99	0.99	1.00	1.00	1.00	1.00	0.01	1.00
T_{max} (hr)	0.25	0.50	0.25	0.50	0.50	0.40	0.14	0.50
C_{max} (mcg/ml)	7.56	6.66	4.73	4.88	5.60	5.89	1.21	5.60
λz (/hr)	0.12	0.04	0.17	0.14	0.18	0.13	0.05	0.13
$t_{1/2\lambda z}$ (hr)	5.70	15.68	4.18	5.14	3.89	6.92	4.95	5.14
$AUC_{0\text{-last}}$ (hr*mcg/ml)	19.31	18.90	14.61	19.83	19.67	18.46	2.18	19.31
$AUC_{0\text{-inf}}$ (hr*mcg/ml)	19.35	18.91	14.63	19.86	19.68	18.49	2.19	19.35
AUC % Extrap (%)	0.21	0.07	0.11	0.15	0.06	0.12	0.06	0.11
$AUMC_{0\text{-last}}$ (hr*mcg*mcg/ml)	68.67	90.07	59.68	71.52	64.91	70.97	11.56	68.67
$AUMC_{0\text{-inf}}$ (hr*mcg*mcg/ml)	70.49	91.85	60.34	72.80	65.38	72.17	12.00	70.49
MRT_{last} (hr)	3.56	4.77	4.09	3.61	3.30	3.86	0.58	3.61
$MRT_{0\text{-inf}}$ (hr)	3.64	4.86	4.13	3.67	3.32	3.92	0.60	3.67
Vd/F (ml/Kg)	935.21	2631.00	908.00	821.01	626.97	1188.04	817.61	908.00
CL/F (ml/hr/Kg)	113.70	116.34	150.42	110.77	111.79	120.60	16.80	113.70

Table 6 Plasma noncompartmental PK parameters for flunixin following administration of 2.2 mg/kg intramuscular to goats. [T_{max} time to maximum concentration; C_{max} maximum plasma concentration; AUC, area under the concentration-time curve; AUMC, area under the first moment-time curve; λ, slope of the terminal phase; $t_{1/2\lambda}$, half -life of terminal phase, Vd/F, volume of distribution; CL/F, total body clearance]. [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

Flunixin in Urine

The median urine C_{max} (15.5 [6.2-17.8] µg/mL) for goats administered flunixin was obtained around 8 hours and declined slowly until approximately 216 hours (Figure 5).

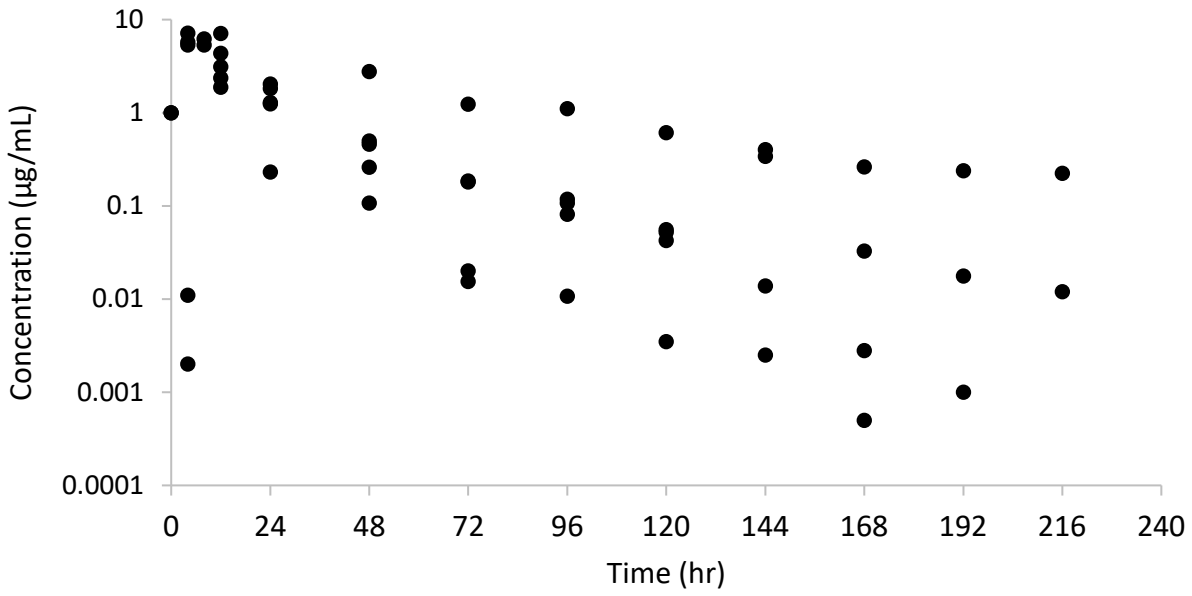

Figure 5 Urine flunixin concentration versus time profile from 5 goats after a single intramuscular dose of flunixin meglumine (2.2 mg/kg). [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

Individual goat PK variables in urine are presented in Table 7. Median AUC in urine was 119.1 [101.3-364.6] µg*hr/mL. All 5 goats had quantifiable levels of flunixin up to day 5 following intramuscular administration, while 2 of the goats had detectable levels of flunixin on day 9 following drug administration. At 240 hours, the urine concentrations for all 5 goats dropped below the LOD (0.5 ng/mL). Renal clearance for flunixin ranged from 5.0 to 9.1 mL/hr/kg (Table 8).

PK Parameter	Goat 1	Goat 2	Goat 3	Goat 4	Goat 5	Mean	SD	Median
T_{max} (hr)	8.00	8.00	8.00	8.00	8.00	8.00	0.00	8.00
C_{max} (mcg/ml)	17.78	17.19	6.19	15.47	7.16	12.76	5.63	15.47
$t_{1/2\lambda z}$ (hr)	52.64	16.85	19.36	12.95	37.91	27.94	16.81	19.36
$AUC_{0\text{-}inf}$ (hr*mcg/ml)	364.55	101.33	119.11	132.94	109.67	165.52	111.88	119.11

Table 7 Urine noncompartmental PK parameters for flunixin following intramuscular administration (2.2 mg/kg). [T_{max} time to maximum concentration; C_{max} maximum urine concentration; AUC, area under the concentration-time curve; $t_{1/2\lambda}$, half -life of terminal phase]. [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

	Flunixin (mL/hr/kg)	Renal Clearance Component of CL/F (%)
Goat 1	9.1	8.2
Goat 2	5.0	4.4
Goat 3	5.6	3.7
Goat 4	7.1	6.1
Goat 5	5.1	4.6

Table 8 Renal clearance values and percent (%) contribution of renal clearance to total systemic clearance (CL/F) for flunixin (n = 5) following intramuscular administration of (2.2 mg/kg). [Mean ± SD = 6.4 ± 1.7] [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

Flunixin in Liver

Liver samples were collected at 360 hours post drug administration. Flunixin was only detected in the liver of goat #4 at a concentration of 8.5 ng/g.

Discussion

Historically, urine has been the sample of choice when drug testing exhibition animals. One objective of this study was to evaluate the effectiveness of plasma as a viable sample for drug testing exhibition animals for flunixin. Flunixin concentrations in plasma were lower than those measured in urine at any given time. Flunixin concentrations in plasma fell below the LOQ in all goats at 60 hours following drug administration, whereas flunixin concentrations in urine were detected for considerably longer (216 hours). Flunixin reached peak plasma concentrations (5.6 [4.7-7.6] µg/mL) in approximately 30 minutes, which is similar to median values reported in lactating dairy goats.[37] The median apparent elimination half-life (5.14 [3.9-15.7] hours) is

similar to the mean reported in dairy goats (2.6-6.8 hours)[37] and other ruminant species including cattle (4.48 hours),[35] and sheep (3.8 hours).[38] The median area under the concentration time curve (19.4 [14.6-19.9] μg*hr/mL) is also similar to the mean reported in dairy goats (16 μg*hr/mL).[37] One question that arises when trying to interpret the detection of flunixin in exhibition animals is the duration of performance-enhancing effects. Unfortunately, the relationship between the pharmacokinetics (*e.g.*, plasma and urine concentrations) and pharmacodynamics (*e.g.*, drug effect in the animal) of flunixin has been difficult to establish in food animals.[37] Measurement of serum thromboxane (TXB_2), a biomarker for NSAIDs exposure, has been performed following flunixin administration in sheep,[39] goats,[40] and calves.[41,42] However, urine biomarkers of flunixin exposure in food animals have not been described. Given that PD data for flunixin in plasma is available, and the shorter amount of time flunixin is detected in plasma compared to urine, plasma may provide a stronger correlation to the performance-altering effects of flunixin in goats. For example, the effects of flunixin administered to horses can persist for up to 30 hours, with maximal effects occurring between 2 and 16 hours.[43] This is one reason that regulation of flunixin in horseracing is performed using plasma, with the regulatory threshold level set at 20 ng/mL and a withdrawal interval of 32 hours.[44] Calves treated with flunixin as an adjunctive therapy for respiratory disease had a reduced respiratory rate and body temperature in as little as 6 hours, with clinical effects persisting for up to 24 hours.[45,46] It is not likely the performance-enhancing effects of flunixin in goats will persist for 9 or more days, when urine concentrations can still be detected. Given that plasma may correlate more strongly with the performance-altering effects of flunixin in goats, and the LOQ and LOD in plasma and urine are equivalent, plasma is a viable sample for drug testing exhibition animals for flunixin.

Our second objective was to establish a conservative urine withdrawal time for flunixin administered intramuscular to goats. Taking into consideration the results of this study and the current "zero tolerance" policy adopted by many livestock show regulators, a conservative urine withdrawal time must be longer than 9 days to achieve a flunixin concentration below the LOD of 0.5 ng/mL. A conservative plasma withdrawal time for flunixin, administered intramuscular to goats, can also be obtained from the data should plasma be selected to drug test exhibition animals for flunixin. A conservative plasma withdrawal time must be longer than 108 hours (4.5 days) to achieve a flunixin concentration below the LOD of 0.5 ng/mL. A calculated approach to estimate a withdrawal interval based on performance-altering effects will be discussed in Chapter V.

Our next objective was to describe the renal clearance of intramuscular flunixin in goats. Renal clearance and urine flunixin concentrations in goats are affected by three main processes involved in renal elimination: glomerular filtration, tubular secretion, and tubular reabsorption. The glomerular filtration rate (GFR) in goats, as assessed by inulin clearance, is approximately 2.2 mL/min/kg.[47] The mean renal clearance for flunixin in goats reported in this study (6.4 ± 1.1 mL/hr/kg) is lower than the mean GFR in goats. Glomerular filtration, tubular section, and tubular reabsorption may also contribute to differences in renal elimination of flunixin between species, as well as the percentage of renal contribution to systemic clearance of flunixin. While not examined in this study, some factors that can influence drug excretion due to GFR include renal blood flow and drug protein binding,[47] age,[48,49] and disease state.[50] Elimination of many NSAIDs is predominantly via hepatic biotransformation. Renal excretion of unmetabolized NSAIDs is usually small (<5% of the dose).[51] Renal clearance values reported in Table 8 were determined based on urine collected for more than 5 half-lives. The amount of flunixin

eliminated via renal excretion as a percent of the total dose administered was low, ranging from 3.7 to 8.2%. This suggests that the primary route of elimination of flunixin in goats is via hepatic metabolism to more polar metabolites, such as 5-hydroxy flunixin,[52] similar to camels,[27] and horses.[53] However, we did not measure metabolites in urine or feces. Systemic clearance (CL/F) in goats administered flunixin (120.6 ± 16.8 mL/hr/kg) is lower compared to values reported in cattle (150.6 ± 43.1 mL/hr/kg).[35]

Our last objective was to correlate flunixin concentrations in urine and liver. Drug distribution in the body is affected by its binding to plasma proteins and tissue.[47] The liver, one of the primary organs responsible for drug metabolism and elimination, is considered an edible tissue and is therefore a key component when assessing food safety. Regulators set tolerances for edible tissues to protect consumers. As discussed in Chapter I, the tolerance is the maximum concentration of a drug allowed in edible tissues from food-producing animals for human consumption. Determination of tolerances for edible tissues is performed following regulations established by the FDA Center for Veterinary Medicine (CVM).[54] Briefly, toxicology studies are performed to determine the acceptable daily intake (ADI) of residues in edible tissues. The ADI is based on the highest dose of a drug that produces no observable effects, which can be a no-observed-effect level (NOEL) or a no-observed-adverse-effect level (NOAEL). A safety factor, which reflects uncertainties associated with the extrapolation of data and information from toxicology studies to humans, is then applied. Additional residue chemistry studies are performed to measure residue depletion and identify marker residues and target tissues. The tolerance for flunixin in bovine liver is 125 ppb in the US (FDA) and 300 ppb in Europe (European Union).[55] The FSIS quantitative test for flunixin in bovine liver has an LOD ≥ 62.5 ppb.[56] Only one goat (#4) had a detectable level of flunixin (8.5 ng/g) in liver upon collection at

360 hours post drug administration. This concentration is considerably below the US and European tolerances, as well as the FSIS LOD. However, flunixin is not approved for use in goats and thus any concentration detected in tissue by FSIS will result in a violation. Data describing flunixin distribution and binding to tissue in goats are limited. In some instances, livestock shows try to interpret and correlate drug concentrations in urine with tissue withdrawal times and tolerances. Because liver was only collected at one time point, it was not possible to assess the correlation between flunixin concentrations in urine and liver over time. Scientifically-based recommendations regarding safe withdrawal intervals of drugs and chemicals in food-producing animals is performed by the Food Animal Residue Avoidance Databank (FARAD), a component of the Food Animal Residue Avoidance & Depletion Program. The recommended meat withdrawal interval, provided by FARAD, in goats following intramuscular administration of a single dose (2.2 mg/kg) of flunixin is 15 days.[57]

Finally, it appears that goat #4 is an outlier. Goat #4 had a prolonged plasma elimination half-life (15.7 hours) and much larger volume of distribution compared to the other goats. Goat #4 produced considerably less urine than the other goats over the course of sample collection. This animal also had a urine flunixin concentration of 224 ng/mL on day 9. This level represents a high concentration when compared to the quantitative flunixin data presented in Figure 3 (Chapter II), and is unexpected considering the amount of time that passed following a single intramuscular dose. Additionally, goat #4 was the only goat with a detectable flunixin concentration in liver at 360 hours following drug administration. This outlier may have had a decreased rate and extent of drug absorption compared to the other goats. Bioavailability of intramuscular administration of flunixin has been shown to be variable in ruminants, ranging from 76% in cattle[58] to 79% in goats.[37] Second, impaired renal function could have led to

decreased renal clearance and reduction in urine output. Tests to monitor hepatic and renal function were not performed during this study, so it is not known if this goat had decreased renal function that may have affected the elimination rate of flunixin.

Conclusions

While blood collection is more invasive than urine collection, and some food animals are more difficult to bleed (*e.g.*, pigs), flunixin concentrations in plasma likely correlate more strongly with potential performance-enhancing effects in exhibition animals. Additionally, testing plasma in goats may allow a shorter conservative withdrawal time following a single intramuscular dose of flunixin compared to urine, as flunixin was detected for a shorter amount of time in plasma compared to urine. Consideration should be given to testing both plasma and urine in exhibition animals, similar to doping control in horseracing.[44] Plasma may be better suited for testing for therapeutic drugs while urine offers a much longer detection time and is more appropriate for prohibited substances.

A conservative urine withdrawal time to meet current "zero tolerance" policy has been proposed. However, as previously mentioned, it is unlikely flunixin provides an unfair advantage to a goat 9 days after it was administered. Therefore, decision limits should be established for therapeutic drugs detected in plasma and urine from exhibition animals. Establishing decision limits for therapeutic drugs in exhibition animals will be discussed in Chapter V.

The mean renal clearance for flunixin in goats reported in this study (6.4 ± 1.1 mL/hr/kg) is lower than the mean GFR in goats (132 mL/hr/kg).[47] The amount flunixin that was eliminated by the kidney was small. This suggests the primary route of elimination of flunixin in goats is via hepatic metabolism.

A common question that is posed when a urine sample from a goat tests positive for flunixin is one about food safety and estimating tissue withdrawal time based on the urine concentration. From the data obtained in this study, it is not possible to make this correlation. Liver samples were only collected once, at the end of the study, and only one goat had a detectable level of flunixin in its liver. Additionally, the conservative urine and plasma withdrawal times proposed in this study should not be used to estimate a tissue withdrawal time for flunixin in goats. Additional residue chemistry studies should be performed evaluating flunixin concentrations in liver samples collected from goats over time to determine an appropriate tissue withdrawal time.

CHAPTER IV

PLASMA PHARMACOKINETICS AND RENAL CLEARANCE OF MELOXICAM IN GOATS*

Introduction

Meloxicam (*e.g.*, Metacam®) is a nonsteroidal anti-inflammatory drug indicated for alleviation of inflammation and relief of pain in both acute and chronic musculo-skeletal disorders in dogs and cats.[59] Meloxicam works by blocking cyclooxygenase (COX), the enzyme responsible for converting arachidonic acid into prostaglandin H_2, the first step in the synthesis of prostaglandins.[60] Prostaglandins, among other actions, are involved in inflammation through their role in vasodilation. Meloxicam has been shown to block cyclooxygenase-2 (COX-2) more than cyclooxygenase-1 (COX-1),[61] making it desirable for treating pain and inflammation without the adverse effects of blocking COX-1, such as mucosal damage leading to ulceration throughout the gastrointestinal tract.[62]

Although not labeled for food animals, meloxicam has been shown to be effective in treating and managing pain and inflammation in cattle,[63-67] sheep,[68-71] goats,[72] and pigs.[73] It may be dosed less frequently than flunixin. For example, a single subcutaneous dose of meloxicam has been shown to be as clinically effective as up to 3 consecutive daily intravenous doses of flunixin meglumine when used as an adjunctive therapy in the treatment of acute febrile respiratory disease in feedlot cattle.[74] While meloxicam is indicated for therapeutic use, it can also be used to alter the performance of an animal by masking pain during competition. As

*Reprint of published data from Small Rumin. Res. 2019. 174:40-46.

previously described in Chapters II and III, regulation of therapeutic drugs like meloxicam is complicated and a “zero tolerance” approach may be too stringent.

There are currently no NSAIDs approved by the FDA for small ruminants. Meloxicam can be used extralabel only if prescribed by a licensed veterinarian in the context of a valid veterinarian-client-patient relationship and all other requirements of the Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1994.[33] Additionally, a tolerance for meloxicam in goats does not exist. Thus, detection of any amount of meloxicam in edible tissues from goats at harvest will result in a violation of federal law. While the Food Safety Inspection Service (FSIS) tests tissues from carcasses for illegal drug residues, urine is often collected at livestock show exhibitions and tested for performance-altering substances. Urine concentrations of meloxicam have been evaluated in horses,[75] greyhounds,[76] camels,[77] cats,[78] dogs,[79] and humans.[80] However, little is known about meloxicam concentrations detected in urine from goats, and how these levels correlate to plasma and tissue concentrations.

Pharmacokinetic (PK) studies are performed to evaluate the movement of a drug into, throughout and out of the body, that is, the time course of its absorption, bioavailability, distribution, metabolism, and excretion.[32] These studies usually involve the collection of multiple plasma, urine, and in some cases tissue, samples over time. There are currently no published PK studies for evaluating the correlation between plasma, urine and tissue concentrations of meloxicam in goats.

PK studies provide data that allow regulators to modify rules as drug testing platforms change. These studies can also aid veterinarians by enabling them to provide guidance to their livestock show clients and avoid jeopardizing their ability to exhibit an animal at the expense of the animal’s health. The objectives of this study were to describe plasma and urine PK including

renal clearance of meloxicam administered orally in goats, to evaluate the effectiveness of plasma as a viable sample for drug testing in exhibition animals, and to estimate a conservative urine withdrawal time.

Materials and Methods

Animals and Housing

Six (6) castrated male Boer goats, between 5 to 8 months old and weighing 22.2 to 36.4 kg at the time of dosing, were used for this study. Each animal was housed in individual metabolism cages. The metabolism cage dimensions (length by width by height) were as follows: 5.5 x 2.5 x 3.5 feet and were specifically designed for small ruminant metabolism research. The cages were cleaned prior to the study and configured with the correct sizing of the head gates to allow the goats free access to water, feed, and hay. Each cage was separated by a minimum of 3 feet. The goats were placed in the metabolism cages approximately 24 hours prior to dosing to allow them to acclimate. The goats were fed a commercial goat feed (Purina Mills) twice daily with free access to water and coastal Bermuda grass hay *ad libitum* throughout the study. Each goat received a physical examination prior to the study, and clinical examinations of all body systems were unremarkable, although liver and kidney function tests were not performed prior to the start of the study. Given that these organs are important for drug clearance, any underlying variability in organ function may be reflected in variability in pharmacokinetic parameter estimates from this study. None of the goats had any previous history of disease or prior exposure to NSAIDs before beginning the study.

Drug Administration and Blood Collection

The goats were weighed on a digital scale the morning of study commencement to determine the appropriate administration dose. Approximately 24 hours prior to dosing, the goats

were restrained and sedated for intravenous catheter placement. The area where the catheter was inserted was clipped and scrubbed with alternating swabs of chlorhexidine and isopropyl alcohol. Using sterile technique, a 16 G x 3.25 in. (Angiocath™, BD, Franklin Lakes, NJ, USA) was inserted into the right jugular vein with an extension set and sutured to the skin using 2-0 monofilament suture. Catheters were flushed twice daily with 6 mL of 10 units/mL heparin saline. An oral dose of meloxicam (0.5 mg/kg; 15 mg tablets, Cipla USA Inc., Sunrise, FL) was administered to each goat per the labeled instructions. The meloxicam tablets were dissolved in 5 mL of water and administered using a 60 mL catheter tipped syringe. Blood samples were taken from the jugular vein and were transferred to lithium heparinized tubes at 0 (pretreatment), 0.25, 0.5, 1, 2, 6, 10, 18, 36, 60, 84, 96, 120, 132, 144, 156, 180, 204, and 228 hours post administration of meloxicam.[34] The samples were stored on ice until centrifugation. The samples were centrifuged at approximately 4,500 g for 10 minutes to yield plasma. The plasma samples were then stored at -80°C until analysis.

Urine Collection

Urine was collected by placing stainless steel buckets beneath the floor of the metabolism cages. A wire mesh was placed across the top of the buckets to prevent feces contamination of the urine. Urine was collected at 0 (pretreatment), 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 336, and 360 hours following drug administration.[34] The total volume of urine produced was measured and recorded at each time point. The buckets were cleaned between each sampling to prevent contamination and sample dilution. The pH of the urine was also measured and recorded at each time point using pH paper (Hydrion, Micro Essential Lab, Inc. Brooklyn, New York). Each urine sample represents fluid collection over time (*e.g.*, not an instantaneous sample) therefore, the total amount of drug excreted at each time point was

calculated using the total urine volume measured for each sample. Urine samples were stored at -80°C until analysis.

Postmortem Tissue Collection

Each goat was humanely euthanized after 360 hours following sedation with xylazine using an intravenous dose of 1 mL/10 lbs. of pentobarbital sodium and phenytoin sodium. Approximately 10 grams of liver were collected from the caudal lobe on the right side of each goat.[34] Samples were stored at -80°C until analysis.

Drug Analysis

Plasma and urine samples were analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) following protein precipitation.[34] A reference standard for meloxicam (purity >99%) was purchased from Sigma Aldrich. The LC-MS/MS method was validated using blank plasma and urine from goats not enrolled in the study. Plasma samples were thawed and extracted using a previously described method.[35] Briefly, 100 µL of each sample was combined with 500 µL of 0.5% citric acid in acetonitrile. Samples were vortexed for approximately 10 seconds and then centrifuged at 4500 RPM for 5 minutes. The supernatant from each sample was transferred into a clean glass borosilicate (16 mm x100 mm) culture tube. The supernatant was then dried via Rapid Vac Evaporation at 55°C under a stream of nitrogen (24 psi) for approximately 15 minutes. Each sample was reconstituted in 300 µL of 50:50 acetonitrile:deionized water (ddH_2O) with 0.1% formic acid, and vortexed for approximately 30 seconds. The samples were then filtered through a 0.2 µ PTFE Whatman Mini-uniprep syringe filter and analyzed by LC-MS/MS. The injection volume was 5 µL for all samples. The mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile at a flow rate of 0.4 mL/min. A gradient was applied beginning at 35% B increasing to 90% B in 1

minute, maintained at 90% B for 1.1 minutes, followed by re-equilibration to 35% B for 1.5 minutes. Chromatographic separation was achieved using an HSS 1.8 µm 2.1 x 100 mm column (Waters, Milford, MA) and maintained at 40°C. Meloxicam eluted at 2.38 minutes. Calibration standards were prepared over a linear range for each sample matrix. The calibration solution ranged from 0.5 to 500 ng/mL for plasma and urine. The LOQ and LOD were determined to be 5 ng/mL and 1 ng/mL, respectively. The run time was 5 minutes for each injection.

PK Analysis

Drug concentrations were analyzed using commercially available software (Phoenix® WinNonlin® version 6.3; Certara USA, Inc., Princeton, NJ, USA) to determine PK parameters for each goat. Noncompartmental analysis (weighted $1/Y^2$) was used to determine the terminal rate constant (λ_z), half-life of the terminal phase ($t_{1/2\lambda}$), and total area under the curve (AUC) for meloxicam (PO). The maximum concentrations (C_{max}) and time to maximum concentration (T_{max}) in plasma and urine were also determined for each goat. Individual renal clearance values, corrected for body weight, were estimated for each goat using the following equation[36]:

$$Renal\ Clearance\ (mL/hr)\ = \left(\frac{Ae}{AUC} * BW\right);$$

where Ae is the cumulative amount of drug excreted unchanged in the urine up to 360 hours, AUC is the area under the plasma drug concentration versus time curve to infinity, and BW is the body weight (kg) of each individual goat.

Results

The original data was collaboratively obtained and shared between two different institutions. Manipulation of the data was performed independently. One form of the data has been reported.[34]

Meloxicam in Plasma

Median plasma meloxicam concentrations over time following a single oral dose of 0.5 mg/kg are presented in Figure 6.

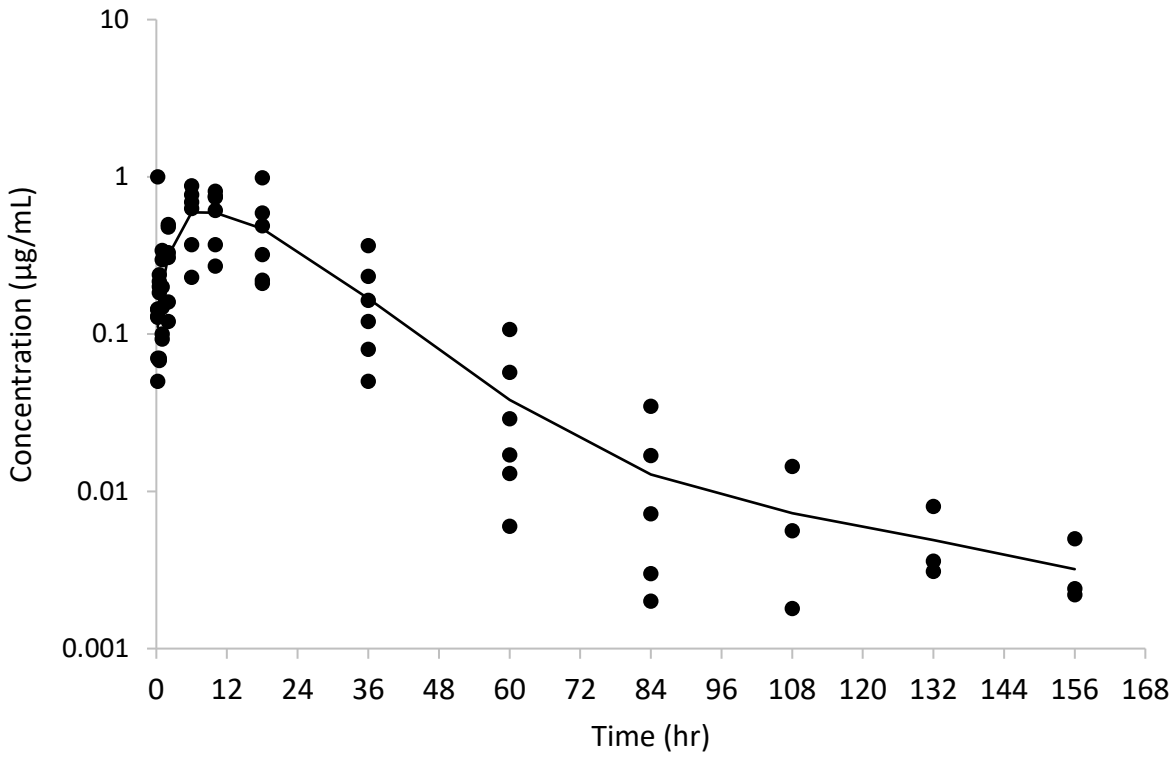

Figure 6 Plasma meloxicam concentration versus time profile from 6 goats after a single oral dose of meloxicam (0.5 mg/kg). Note: Plasma data are expressed as individual points; the solid line represents the mean of all goats. [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

Individual plasma PK parameters for goats administered meloxicam are presented in Table 9. Meloxicam plasma concentrations after oral administration fell below 0.01 µg/mL after 150 hours in all 6 goats.

PK Parameter	Goat 1	Goat 2	Goat 3	Goat 4	Goat 5	Goat 6	Mean	SD	Median
r^2	0.99	1.00	1.00	0.97	1.00		0.99	0.01	1.00
T_{max} (hr)	6.00	10.00	6.00	6.00	18.00	6.00	8.67	4.84	6.00
C_{max} (mcg/ml)	0.37	0.27	0.63	0.77	0.99	0.88	0.65	0.28	0.70
λz (/hr)	0.10	0.08	0.07	0.02	0.02		0.06	0.04	0.07
$t_{1/2\lambda z}$ (hr)	7.04	9.17	9.55	39.33	32.82		19.58	15.26	9.55
$AUC_{0\text{-last}}$ (hr*mcg/ml)	8.59	7.30	14.91	23.12	33.12	20.39	17.91	9.73	17.65
$AUC_{0\text{-inf}}$ (hr*mcg/ml)	8.66	7.33	14.95	23.25	33.36		17.51	10.87	14.95
AUC % Extrap (%)	0.70	0.36	0.28	0.54	0.71		0.52	0.20	0.54
$AUMC_{0\text{-last}}$ (hr*mcg*mcg/ml)	145.71	132.63	247.61	523.25	869.58	383.95	383.79	280.62	315.78
$AUMC_{0\text{-inf}}$ (hr*mcg*mcg/ml)	149.98	135.20	251.66	549.81	917.72		400.87	333.58	251.66
MRT_{last} (hr)	16.95	18.17	16.61	22.63	26.26	18.83	19.91	3.78	18.50
$MRT_{0\text{-inf}}$ (hr)	17.33	18.45	16.83	23.65	27.51		20.75	4.65	18.45
Vd/F (ml/Kg)	586.52	903.36	460.72	1220.38	709.72		776.14	297.23	709.72
CL/F (ml/hr/Kg)	57.77	68.25	33.44	21.51	14.99		39.19	23.03	33.44

Table 9 Plasma noncompartmental PK parameters for meloxicam following oral administration (0.5 mg/kg). [T_{max} time to maximum concentration; C_{max} maximum plasma concentration; AUC, area under the concentration-time curve; AUMC, area under the first moment-time curve; λ, slope of the terminal phase; $t_{1/2\lambda}$, half -life of terminal phase, Vd/F, volume of distribution; CL/F, total body clearance] [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

Meloxicam in Urine

Individual urine PK parameters for goats administered meloxicam are presented in Table 10. Urine concentrations for goats administered meloxicam were first detected in urine by 4 hours post dose (Figure 7). After 100 hours, all urine samples fell below 100 ng/mL and at 216 hours, meloxicam concentrations in urine from all 6 goats were below the LOD. One goat had considerably lower urine output, which was likely the reason for prolonged drug detection. The median terminal elimination half-life in urine was 35.82 [15.4-58.2] hours.

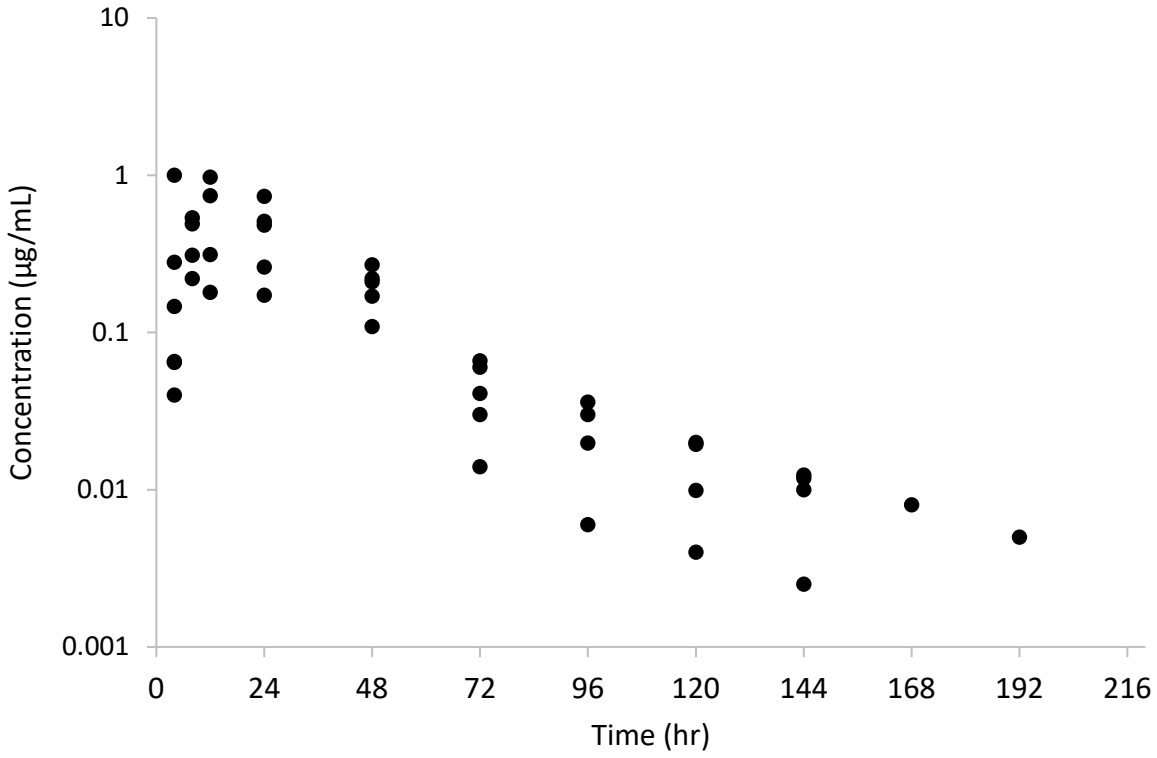

Figure 7 Urine meloxicam concentration versus time profile from 6 goats following oral administration of meloxicam (0.5 mg/kg). [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

Renal clearance for meloxicam ranged from 0.2 to 2.0 mL/hr/kg and percent contribution of renal clearance to total clearance was low, ranging from 1.0 to 5.0% (Table 10).

PK Parameter	Goat 1	Goat 2	Goat 3	Goat 4	Goat 5	Goat 6	Mean	SD	Median
T_{max} (hr)	12.00	12.00	24.00	24.00	12.00	12.00	16.00	6.20	12.00
C_{max} (mcg/ml)	0.41	0.74	0.73	0.26	0.31	0.97	0.57	0.28	0.57
$t_{1/2\lambda z}$ (hr)	58.21	34.08	29.55	15.41	37.56	38.00	35.47	13.91	35.82
$AUC_{0\text{-}inf}$ (hr*mcg/ml)	16.42	26.38	30.33	13.65	12.19	23.88	20.48	7.42	20.15

Table 10 Urine noncompartmental PK parameters for meloxicam following oral administration (0.5 mg/kg). [T_{max} time to maximum concentration; C_{max} maximum urine concentration; AUC, area under the concentration-time curve; $t_{1/2\lambda}$, half -life of terminal phase] [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

	Meloxicam (mL/hr/kg)	Renal Clearance component of CL/F (%)
Goat 1	0.2	1.0
Goat 2	2.0	2.9
Goat 3	0.5	3.1
Goat 4	0.5	1.5
Goat 5	1.1	5.0
Goat 6	1.5	2.6

Table 11 Renal clearance values and percent (%) contribution of renal clearance to total systemic clearance (CL/F) for meloxicam (n = 6) following oral administration of (0.5 mg/kg). [Mean ± SD = 1.0 ± 0.7] [Reprinted from Small Rumin. Res. 2019. 174:40-46.]

Meloxicam in Liver

Meloxicam was not detected in the liver in 2 of 6 goats at 336 hours. Three (3) goats had detectable levels of meloxicam (1.4, 1.8, and 1.5 ng/g) in liver upon collection at 360 hours post drug administration.

Discussion

Historically, urine has been the sample of choice when drug testing exhibition animals. One objective of this study was to evaluate the effectiveness of plasma as a viable sample for drug testing exhibition animals for meloxicam. The median C_{max} for meloxicam was similar between plasma (0.7 [0.27-0.99] µg/mL) and urine (0.57 [0.26-0.97] µg/mL). Plasma concentrations of meloxicam were below the LOD (1 ng/mL) in all goats at 180 hours (7.5 days) post drug administration. Urine concentrations of meloxicam were below the LOD in all goats at 216 hours (9 days) post drug administration. Similar to flunixin, one question that arises when

trying to interpret the detection of meloxicam in exhibition animals is the duration of performance-enhancing effects. The relationship between the pharmacokinetics (*e.g.*, plasma and urine concentrations) and pharmacodynamics (*e.g.*, drug effect in the animal) of meloxicam in food animals is not well described. Measurement of serum thromboxane (TXB_2) has been performed following meloxicam administration in piglets.[81] However, urine biomarkers of meloxicam exposure in food animals have not been described. The efficacy of meloxicam in horses has been shown to last for several days. For example, the effects of meloxicam administered to horses following orthopedic surgery persisted for 6 days, following daily dosing for 5 days.[82] Meloxicam has also been shown to significantly reduce the display of painful behaviors and physiological responses to pain in band castrated and surgical castrated calves for up to 72 hours following a single oral treatment.[83] It is unlikely the performance-enhancing effect of meloxicam in goats will persist for 8 days following a single oral dose, when urine concentrations can still be detected. Therefore, plasma may correlate more strongly to the performance-altering effects of meloxicam in goats, given the shorter amount of time meloxicam is detected in plasma compared to urine. Given that plasma may correlate more strongly to the performance-altering effects of meloxicam in goats, and the LOQ and LOD in plasma and urine are equivalent, plasma is a viable sample for drug testing exhibition animals for meloxicam.

Our second objective was to establish a conservative urine withdrawal time for meloxicam administered orally to goats. Taking into consideration the results of this study and the current "zero tolerance" policy adopted by many livestock show regulators, a conservative urine withdrawal time must be longer than 8 days to achieve a meloxicam concentration below the LOD of 1 ng/mL. A conservative plasma withdrawal time for meloxicam administered orally to goats can also be obtained from the data, should plasma be selected to drug test exhibition

animals for meloxicam. A conservative plasma withdrawal time must be longer than 156 hours (6.5 days) to achieve a meloxicam concentration below the LOD of 0.5 ng/mL. A calculated approach to estimate a withdrawal interval based on performance-altering effects will be discussed in Chapter V.

Our next objective was to describe the renal clearance of oral meloxicam in goats. Renal clearance and urine meloxicam concentrations in goats are affected by three main processes involved in renal elimination: glomerular filtration, tubular secretion, and tubular reabsorption. The glomerular filtration rate (GFR) in goats, as assessed by inulin clearance, is approximately 2.2 mL/min/kg.[47] The mean renal clearance for meloxicam in goats reported in this study (1.0 ± 0.7 mL/hr/kg) is lower than the mean GFR in goats. Glomerular filtration, tubular section, and tubular reabsorption may also contribute to differences in renal elimination of meloxicam between species, as well as the percentage of renal contribution to systemic clearance of meloxicam. While not examined in this study, some factors that can influence drug excretion due to GFR include renal blood flow and drug protein binding,[47] age,[48,49] and disease state.[50] Elimination of many NSAIDs is predominantly via hepatic biotransformation. Renal excretion of unmetabolized NSAIDs is usually small (<5% of the dose).[51] Renal clearance values reported in Table 11 were determined based on urine collected for more than 5 half-lives. The amount of meloxicam eliminated via renal excretion as a percent of the total dose administered was low, ranging from 0.9 to 4.6%. This suggests that the primary route of elimination of meloxicam in goats is via hepatic metabolism to more polar metabolites, such as 5'-hydroxymethylmeloxicam and 5'-carboxymeloxicam, similar to rats,[84,85] mice,[85] mini-pigs,[85] humans,[85,86] and horses.[87-89] However, we did not measure metabolites in urine or feces.

Our last objective was to correlate meloxicam concentrations in urine and liver. Drug distribution in the body is affected by its binding to plasma proteins and tissue.[47] The liver, one of the primary organs responsible for drug metabolism and elimination, is considered an edible tissue and is therefore a key component when assessing food safety. Regulators set tolerances for edible tissues to protect consumers.[54] As discussed in Chapter I, the tolerance is the maximum concentration of a drug allowed in edible tissues from food-producing animals for human consumption. A tolerance for meloxicam has not been established in edible tissues for goats. The FSIS quantitative test for meloxicam in bovine kidney and muscle has an LOD $\geq$ 10 ppb.[56] Three (3) goats had detectable levels of meloxicam (1.4, 1.8, and 1.5 ng/g) in liver upon collection at 360 hours post drug administration. This concentration is considerably below the FSIS LOD for meloxicam in bovine kidney and muscle. However, meloxicam is not approved for use in goats and thus any concentration detected in tissue by FSIS will result in a violation. Data describing meloxicam distribution and binding to tissue in goats are limited. In some instances, livestock shows try to interpret and correlate drug concentrations in urine with tissue withdrawal times and tolerances. Because liver was only collected at one time point, it was not possible to assess the correlation between meloxicam concentrations in urine and liver over time.

Conclusions

Similar to flunixin, meloxicam concentrations in plasma likely correlate more strongly with potential performance-enhancing effects in exhibition animals. Additionally, testing plasma in goats may allow a shorter conservative withdrawal time following a single oral dose of meloxicam compared to urine, as meloxicam was detected for a shorter amount of time in plasma compared to urine. However, consideration should be given to testing both plasma and urine in

exhibition animals. Plasma may be better suited for testing for therapeutic drugs, but urine is likely more appropriate for prohibited substances.

A conservative urine withdrawal time of longer than 8 days is necessary to meet current "zero tolerance" regulations. However, as previously mentioned, it is unlikely meloxicam provides an unfair advantage to a goat 8 days after it was administered.

The mean renal clearance for meloxicam in goats reported in this study (1.0 ± 0.7 mL/hr/kg) is lower than the mean GFR in goats (132 mL/hr/kg).[47] The amount of meloxicam that was eliminated by the kidney was small. This suggests the primary route of elimination of meloxicam in goats is via hepatic metabolism.

It was not possible to make a correlation between urine and tissue concentrations of meloxicam. Liver samples were only collected once, at the end of the study, and while three goats had detectable levels of meloxicam in liver, the levels were quite low. Therefore, the conservative urine and plasma withdrawal times proposed in this study should not be used to estimate a tissue withdrawal time for meloxicam in goats.

CHAPTER V

INTEGRATION OF OBSERVED AND PUBLISHED DATA, PK/PD CONCEPTS, AND MEASUREMENT UNCERTAINTY TO PROPOSE A METHOD FOR ESTABLISHING DECISION LIMITS FOR THERAPEUTIC DRUGS DETECTED IN ANIMALS EXHIBITED AT LIVESTOCK SHOWS

Introduction to the Problem

Testing for drugs that have the ability to enhance performance in competition animals is common practice. For example, drug testing is performed in many different animal competitions that include: Quarter Horse, Thoroughbred, and Standardbred (harness) racing; equestrian events including dressage, jumping, draft horse showing, trail riding, cutting, and reigning; greyhound racing; camel racing; pigeon racing; and dog pulling/sledding. Another animal industry that utilizes drug testing is the livestock show industry. Similar to other performance animal competitions, livestock shows utilize drug testing to foster fair competition among competitors and ensure animal welfare. Ante-mortem samples, such as urine, blood, and feces are tested for a variety of drugs that are considered to have the ability to enhance performance in livestock. These drugs include nonsteroidal anti-inflammatory drugs (NSAIDs), beta-adrenergic agonist drugs with repartitioning effects, analgesics, stimulants, sedatives and tranquilizers, antihistamines, and illicit drugs.

Testing livestock exhibited at shows for drugs is more complex than the other performance animal competitions, however. Unlike other performance animal competitions, a rationale for drug testing of exhibited livestock is to ensure food safety. In addition to testing ante-mortem samples for performance-enhancing drugs, post-mortem samples from exhibited

livestock also are tested to protect the food supply. Once a livestock project is completed, the animal will enter the food supply. Exhibition animals that enter the food supply represent a large number of carcasses across the country. In 2004 in Texas alone, it is estimated that more than 14 million pounds of carcasses from livestock projects entered the food supply.[26] Post-mortem samples, such as liver, kidney, and muscle are screened for a variety of drugs that include NSAIDs, beta-adrenergic agonists with repartitioning effects, and antibiotics.

Also unique to livestock shows compared to other performance animal competitions is how drug testing is regulated. Regulation of drugs detected in post-mortem samples from exhibition animals is under the purview of the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) Food Safety Inspection Service (FSIS). The FDA approves tolerances and withdrawal times for meat and other edible tissue as part of approval of drugs for use in food animals. The tolerance is the maximum concentration of a drug allowed in edible tissues from food-producing animals for human consumption, and withdrawal time is the amount of time before a food-producing animal can be harvested following drug administration. Regulation of drugs detected in ante-mortem samples from exhibition animals is under the purview of the board of directors of the individual livestock show or fair, and usually involves a so-called “zero tolerance” policy.[1,2] Regulation of drugs detected in ante-mortem samples can be divided into 2 categories: 1) prohibited substances, and 2) drugs that are administered in a legal manner. While “zero tolerance” is appropriate for prohibited substances, thresholds and decision limits should be established for drugs administered legally.

Establishing decision limits for drugs administered legally to exhibition animals, such as therapeutic drugs like NSAIDs, is a novel approach for regulating drug testing of ante-mortem samples in the livestock show industry. Determination of decision limits should be based on

pharmacokinetics, drug disposition and metabolism in the body, and pharmacodynamics, the drug's effect on the body. Establishing a numerical decision-point to classify a sample as being negative or positive also must account for measurement error, and intra- and inter-individual variance.

Currently, the difference between a sample testing positive or negative is determined by the limit of detection of the analytical assay. In other words, if the analytical instrument can detect the presence of a drug in a sample, the sample is deemed positive. Changes in analytical testing methods in recent years have lowered the limit of detection for drugs used in a legal manner. This has increased the number of positive tests, raising concerns about the policy of "zero tolerance".

Current analytical instrumentation used for drug testing allows the detection of 'trace' amounts of chemical substances, including therapeutic drugs. A 'trace' level can be defined as a pharmacologically-insignificant concentration, or the "Highest No Effect Dose" (HNED).[90] Most drug testing laboratories in the US that test samples from exhibited livestock also perform drug testing for the horse racing industry. They must use highly sensitive analytical instruments to detect therapeutic drugs that have very low regulatory threshold concentrations, as regulated by the Association of Racing Commissioners International (ARCI).[44] For example, the regulatory threshold concentration for dexamethasone adopted by the ARCI is 0.005 nanograms per milliliter (ng/mL), which is equivalent to 5 picograms per milliliter (pg/mL) or 500 femtograms per milliliter (fg/mL).[44] To put this into perspective, one nanogram is equivalent to 10^{-9}, while one picogram is equivalent to 10^{-12}, and one femtogram is equivalent to 10^{-15}. Detection of trace levels of therapeutic drugs in animals exhibited at livestock shows is evident by the low levels of flunixin detected from 2016 to 2017 (Figure 3, Chapter II). Prior to 2015, TVMDL screened

samples from exhibition animals for therapeutic drugs, like flunixin, using either thin layer chromatography (TLC) or enzyme-linked immunosorbent assay (ELISA). The limit of detection (LOD) for flunixin in urine using these test methods was 250 ng/mL and 2 ng/mL, respectively. In 2015, TVMDL transitioned the screening of ante-mortem samples from exhibition animals to liquid chromatography – high-resolution accurate mass spectrometry (LC-HRAMS). As a result, the LOD for flunixin improved to 0.05 ng/mL, a 40-fold difference.

Detection of trace levels of therapeutic drugs in animals exhibited at livestock shows raises 2 main concerns. The first concern relates to the degree to which very low levels of therapeutic drugs provide a performance-enhancing effect in the animal. Animals exhibited at livestock shows are unique compared to other performance animals, as they compete based on appearance rather than physical activity. Therefore, drugs used in exhibition animals to enhance performance do so by altering appearance, such as masking pain, lameness, or inflammation, and improving leanness and muscling. Determining the HNED of a drug used to alter the appearance of an exhibition animal is challenging compared to drugs that are used to alter activity. For example, physiologic responses to exercise can be measured in horses, thus determining a drug's effects on performance potential. Pharmacodynamic (PD) end-points such as heart rate,[91] plasma lactate concentration,[91,92] oxygen uptake level,[91] and stride length[91,93] are some of the physiologic parameters that indicate therapeutic effect of drugs in horses. In addition to lack of ability to detect physical effects of drugs in the context of exhibited livestock, researchers have demonstrated that untreated animals can have detectable levels of therapeutic drugs, like flunixin, from exposure to the drug in the environment. Comingling of treated and untreated horses[23] and pigs[8] resulted in detectable levels of drugs like flunixin in the untreated animals. The ARCI has adopted regulatory decision limits for some substances, like caffeine and

theobromine, because they are commonly found in the environment and can possibly contaminate feed or water.[44]

The second concern with "zero tolerance" when regulating therapeutic drugs that are legally administered is predicting a withdrawal time in urine or plasma to avoid detection of drug. Veterinarians who administer therapeutic drugs to exhibition animals in a legal manner face uncertainty when estimating the appropriate amount of time for drug elimination prior to competition. If exhibition animals are not to be deprived of proper veterinary care, suitable information on the time after administration that therapeutic agents may be detected must be made available to the veterinary profession. While urine and plasma withdrawal times for flunixin and meloxicam in goats have been estimated (see Chapters III and IV), there is a need for a general approach to establishing decision limits for therapeutic drugs used in animals exhibited at livestock shows, similar to what is done for horse racing.[44]

Increased sensitivity achieved by current analytical techniques allows the detection of plasma or urine concentrations of therapeutic drugs for extended periods (*i.e.*, days or weeks) after their administration, at which time there is likely no performance-enhancing effect to the animal.[25] Some researchers estimate negligible drug concentrations with regard to therapeutic effects occur after 4[94] to 5 plasma half-lives.[47] Decision limits, drug concentrations at which negligible therapeutic effects occur, can be used by testing laboratories to classify samples as negative or positive, rather than testing at the LOD for each drug, or a "zero tolerance" approach. Additionally, decision limits for therapeutic drugs can also help alleviate the concerns surrounding a positive test resulting from environmental contamination.

Regulation of drugs detected in animals exhibited at livestock shows can be divided into 2 categories: 1) prohibited substances, and 2) drugs that are administered in a legal manner.

While “zero tolerance” is appropriate for prohibited substances, decision limits should be established for the latter. The objective of this chapter is to integrate observed and published data, PK/PD concepts, and measurement error to propose a method for establishing decision limits for therapeutic drugs detected in animals exhibited at livestock shows. To the authors’ knowledge, this is the first attempt to establish decision limits for therapeutic drugs detected in ante-mortem samples from animals exhibited at livestock shows.

Consideration of Alternatives

Selection of an appropriate method for establishing decision limits for therapeutic drugs detected in animals exhibited at livestock shows involves several factors. First, pharmacokinetics (PK) are required to characterize the time course of drug absorption and disposition. Second, pharmacodynamics (PD) are important as they provide information pertaining to drug effect in the animal. Third, it is important to take into consideration tolerances and withdrawal information for drug residues in tissue. Last, decision limits for therapeutic drugs must be determined for each animal species, and will likely differ among species. This adds a level of complexity for testing laboratories as they will need to adapt analytical testing methods and data review processes to account for these inter-species differences in thresholds. Here, we consider 3 different methods for establishing decision limits for therapeutic drugs detected in exhibition animals: 1) the 95/95 tolerance interval (TI)[95] used in the US by the Racing Medication & Testing Consortium (RMTC) Scientific Advisory Committee (SAC); 2) the irrelevant plasma concentration (IPC) and irrelevant urine concentration (IUC)[96] approach used by regulators in horse racing in Europe; and, 3) a novel approach integrating pharmacokinetics and pharmacodynamics, including predicted drug effects based on the sigmoidal E_{max} model using the Hill equation.

95/95 Tolerance Interval (TI)

The first method considered for establishing decision limits in therapeutic drugs detected in animals exhibited at livestock shows was the 95/95 Tolerance Interval[95] used in the US by the Racing Medication & Testing Consortium (RMTC) Scientific Advisory Committee (SAC). This approach involves the natural logarithmic (ln)-transformation of the normalized mean plasma drug concentration measured at a pre-determined withdrawal interval, and a mathematical constant 'K factor' that is based on the number of animals in the study.

95/95 Tolerance Interval (TI) calculation equation:

$$Mean_{ln} + (SD_{ln} \times K\ factor) = 95/95\ TI,$$

where $Mean_{ln}$ is the natural logarithm (ln)-transformed mean plasma concentration at a pre-determined withdrawal interval, and SD_{ln} is ln-transformed standard deviation of the plasma concentrations at the same pre-determined withdrawal interval. The purpose of the K factor is to prevent the 'oversimplification' that can result from attributing too much importance to values from a limited subset of a population. The European Agency for the Evaluation of Medicinal Products' table of K factors used by the RMTC is presented in Table 12.

n	K
2	26.260
3	7.656
4	5.144
5	4.210
6	3.711
7	3.401
8	3.188
9	3.032
10	2.911
11	2.815
12	2.736
13	2.670
14	2.614
15	2.566
16	2.523
17	2.486
18	2.453
19	2.423
20	2.396

Table 12 European Agency for the Evaluation of Medicinal Products' table of K factors. [n = sample size number and K = mathematical constant based on n][95]

Determination of the 95/95 TI is made after establishing an appropriate withdrawal interval. In horse racing, the RMTC SAC determines an appropriate withdrawal interval based on review of the scientific literature and conventional racetrack applications, investigation of any illicit use, and consideration of a medication's potential to affect performance.[95] The committee then reviews the data from the study horses' samples collected at that time-point, and applies the 95/95 TI to establish a regulatory threshold.

The 95/95 TI method was applied to the flunixin data obtained in Chapter III and results are presented in Table 13. For demonstration purposes, withdrawal intervals of 36 hours and 96 hours were applied to plasma and urine, respectively.

Sample Matrix	Withdrawal Interval	Highest Observed Concentration (ng/mL)	95/95 TI (ng/mL)	Proposed Decision Limit (ng/mL)
Plasma	36 HR	19	123	130
Urine	96 HR	1,109	8,459	8,460

Table 13 95/95 Tolerance Interval (TI) for flunixin in goat plasma and urine using a withdrawal interval of 36 hours (plasma) and 96 hours (urine) following intramuscular drug administration.

The margin of safety associated with the ln-transformation of the data and the K factor is quite large. At a 36-hour withdrawal interval, the highest flunixin concentration observed in plasma was 19 ng/mL. The calculated 95/95 TI based on a 36-hour withdrawal interval is 123 ng/mL. Rounding to the next largest 10, a proposed threshold for flunixin detected in plasma from goats administered flunixin at a dose of 2.2 mg/kg (IM), at a withdrawal interval of 36 hours, is 130 ng/mL. At a 96-hour withdrawal interval, the highest flunixin concentration observed in urine was 1,109 ng/mL. The calculated 95/95 TI based on a 96-hour withdrawal interval is 8,459 ng/mL. Rounding to the next largest 10, a proposed threshold for flunixin detected in urine from goats administered flunixin at a dose of 2.2 mg/kg (IM), at a withdrawal interval of 96 hours, is 8,460 ng/mL.

The 95/95 TI method was also applied to the meloxicam data obtained in Chapter IV and results are presented in Table 14. Similar to flunixin, withdrawal intervals of 36 hours and 96 hours were applied to plasma and urine, respectively.

Sample Matrix	Withdrawal Interval	Highest Observed Concentration (ng/mL)	95/95 TI (ng/mL)	Proposed Decision Limit (ng/mL)
Plasma	36 HR	365	1,578	1,580
Urine	96 HR	36	279	280

Table 14 95/95 Tolerance Interval (TI) for meloxicam in goat plasma and urine using a withdrawal interval of 36 hours (plasma) and 96 hours (urine) following oral drug administration.

At a 36-hour withdrawal interval, the highest meloxicam concentration observed in plasma was 365 ng/mL. The calculated 95/95 TI based on a 36-hour withdrawal interval is 1,578 ng/mL. Rounding to the next largest 10, a proposed threshold for meloxicam detected in plasma from goats administered meloxicam at a dose of 0.5 mg/kg (PO), at a withdrawal interval of 36 hours, is 1,580 ng/mL. At a 96-hour withdrawal interval, the highest meloxicam concentration observed in urine was 36 ng/mL. The calculated 95/95 TI based on a 96-hour withdrawal interval is 279 ng/mL. Rounding to the next largest 10, a proposed threshold for meloxicam detected in urine from goats administered meloxicam at a dose of 0.5 mg/kg (PO), at a withdrawal interval of 96 hours, is 280 ng/mL.

Advantages and Disadvantages

One of the advantages in using the 95/95 TI to establish decision limits for therapeutic drugs detected in animals exhibited at livestock shows is that it allows for a large margin of safety. However, the margin of safety associated with determining the 95/95 TI is largely

affected by the sample size. A larger sample size results in a lower K factor. Determination of the 95/95 TI above included 5 animals for flunixin in plasma and 4 animals for flunixin in urine (Table 13), and 6 animals for meloxicam in plasma and 5 animals for meloxicam in urine (Table 14). It is likely the 95/95 TI presented in Table 13 and Table 14 would be lower had there been more animals in the studies performed in Chapters III and IV. Conversely, studies that based thresholds and decision limits by this or any other method that use small sample sizes are problematic.

Another advantage of using the 95/95 TI to establish decision limits is it provides veterinarians with a withdrawal interval. This withdrawal interval will allow a veterinarian to treat an exhibition animal without fear of generating a positive test result at a show or fair from unintended consequences. However, determining an appropriate withdrawal interval for therapeutic drugs in plasma and urine from exhibition animal species is quite daunting. First, the criteria used by the RMTC SAC in determining an appropriate withdrawal interval are not available to livestock show regulators. Information pertaining to a medication's potential to affect performance in an exhibition animal is difficult to measure and not well described in the scientific literature. Second, unlike horseracing, there is no governing regulatory body for the livestock show industry in the US. The board of directors of the individual show or fair makes policies and decisions regarding drug use. Therefore, it will be challenging for all shows and fairs to come to an agreement on appropriate withdrawal intervals.

Last, implementation of the 95/95 TI to establish a decision limit requires standardization of the dose and route of administration. Standardization of the dose and route of administration may be appropriate for drugs used in a labeled manner. However, this will certainly be challenging for drugs used in an extra-label manner, like flunixin in sheep and goats.

Irrelevant Plasma Concentration/Irrelevant Urine Concentration (IPC/IUC)

The second method considered for establishing decision limits in therapeutic drugs detected in animals exhibited at livestock shows was the irrelevant plasma concentration (IPC) and irrelevant urine concentration (IUC)[96] approach used by regulators in horse racing in Europe. Computation of the IPC and IUC involved the following steps[96]:

1. Calculation of an effective plasma concentration (EPC), which takes into consideration the standard dose (per dosing interval), the bioavailability factor, and the plasma clearance (per dosing interval), where F is the bioavailability factor lying between 0 and 1. Typically the EPC is calculated using intravenous data only so that F = 1, as the EPC is the same regardless of route of drug administration.

$$EPC = \frac{standard\ dose\ (per\ dosing\ interval) x\ F}{plasma\ clearance\ (per\ dosing\ interval)}$$

2. Calculation of the IPC, which is made by dividing the EPC by a safety factor (SF). The selection of an SF is mainly a subjective choice of regulatory officials.

$$IPC = EPC/SF.$$

A default SF of 500 (*i.e.*, 10 x 50) is commonly used, with 50 to transform an effective plasma concentration close to EC_{50} into an ineffective one, and 10 to take inter-individual variability into account.[97]

3. The IUC is calculated by multiplying the IPC by the steady-state urine to plasma concentration ratio (Rss).

$$IUC = IPC \times Rss.$$

4. Checking the appropriateness of IPC and IUC. The appropriateness of the IPC and IUC are determined by computing the amount of drug remaining in the body when the plasma concentration is equal to the IPC and by calculating the shortest possible withdrawal time

(WT) of the drug. The residual amount (RA) of drug in the body when plasma concentration is equal to the IPC is given by the following equation:

$$RA = IPC \times Varea,$$

where Varea is the volume of distribution calculated by the area method. This RA can then be compared to the recommended dosage regimen and should be lower than a given percentage of the recommended dose (*e.g.*, 1%).

Results of applying the IPC/IUC method using the flunixin and meloxicam data obtained in Chapters III and IV are presented in Table 15 and Table 16, respectively.

EPC (ng/mL)	IPC (ng/mL)	IUC (ng/mL)
610	1.2	17.7

Table 15 Effective plasma concentration (EPC), irrelevant plasma concentration (IPC) and irrelevant urine concentration (IUC) for flunixin in goats following intramuscular administration (2.2 mg/kg).

EPC (ng/mL)	IPC (ng/mL)	IUC (ng/mL)
305	0.6	0.8

Table 16 Effective plasma concentration (EPC), irrelevant plasma concentration (IPC) and irrelevant urine concentration (IUC) for meloxicam in goats following oral administration (0.5 mg/kg).

Determination of the EPC utilizes a bioavailability factor (F) that lies between 0 and 1. In most cases, the EPC is calculated using intravenous data so that F = 1, as the EPC is the same regardless of the route of drug administration.[96] Even though the flunixin and meloxicam data obtained were not from intravenous drug administration, an F of 1 was still applied when calculating the EPC. Applying an SF of 500 resulted in an IPC of 1.2 ng/mL and IUC of 17.7 ng/mL for flunixin, and an IPC of 0.6 ng/mL and IUC of 0.8 ng/mL for meloxicam. The mean Rss was determined using the urine and plasma drug concentrations at the elimination half-life for each goat. The Rss for flunixin was determined to be 14.4 and the Rss for meloxicam was determined to be 1.3. The RA for flunixin was 1.0 µg/kg, which is below 0.05% of the recommended dose. The RA for meloxicam was 0.32 µg/kg, which is below 0.1% of the recommended dose.

When comparing the flunixin concentrations in urine presented in Figure 3 (Chapter II) to the IUC for flunixin in Table 15 above, one can see the effectiveness of IPC/IUC in establishing a decision limit for flunixin. Out of the 25 animals with quantified flunixin concentrations, 18 were below the IUC of 17 ng/mL. Although the animal species for those values presented in Figure 3 (Chapter II) were not solely goats, a decision limit of 17 ng/mL would have resulted in a negative test result for more than 70% of those animals.

Advantages and Disadvantages

Determination of the IPC and IUC involves plasma clearance, and determination of plasma clearance depends on route of drug administration. One advantage of the IPC/IUC method is its independence from route of administration (providing its action is systemic).[96] However, the IPC/IUC method also relies on the assumption that drug effects are reversibly

driven by the plasma concentration profile, and cannot be used for drugs whose effects last long after the drug has been eliminated (*e.g.*, corticosteroids and anabolic steroids).[96]

Another advantage of using the IPC/IUC method to establish thresholds for therapeutic drugs detected in exhibition animals is that it does not involve a withdrawal interval such that it may be more practicable than the 95/95 TI method to establish decision limits for therapeutic drugs detected in animals exhibited at livestock shows. Another advantage of using the IPC/IUC method to establish decision limits for therapeutic drugs detected in animals exhibited at livestock shows is PK data, such as plasma clearance, used to calculate EPC is readily obtainable from the scientific literature for various exhibition animal species.

A disadvantage of using the IPC/IUC method to establish decision limits for therapeutic drugs detected in exhibited livestock is that this method does not account for a withdrawal interval. Veterinarians face challenges when administering therapeutic drugs to exhibition animals as some drugs are detected in urine after the labeled meat withdrawal time. Using the IPC/IUC method does not provide veterinarians with a withdrawal interval to avoid an adverse analytical finding in urine, from unintentional consequences.

Another disadvantage of this method is that IUC requires knowing the urine to plasma concentration ratio (Rss), which is seldom reported in the scientific literature. Consequently, the Rss must be approximated from published raw data or from published figures, and these may not always be available/accessible.

Sigmoidal E_{max} Model Using the Hill Equation

One of the challenges when therapeutic drugs are detected in urine from animals exhibited at livestock shows is interpreting physiologic or performance-enhancing effects. While

it is difficult to measure performance-enhancing effects of therapeutic drugs in food animals, determination of decision limits should account for drug effect (PD) in the animal. Therefore, the third method we considered for establishing decision limits for therapeutic drugs detected in animals exhibited at livestock shows was the Hill equation, also known as the sigmoidal E_{max} model. The Hill equation is often used in PK-PD modeling to construct the dose-response relationship between drug effect (often the concentration of some biomarker(s)) and drug concentration given by the following equation[39,98]:

$$E = \frac{E_{max}\, x\, C^n}{C^n + IC_{50}{}^n};$$

where E is the drug effect expressed in percentage inhibition, E_{max} is the maximal inhibitory effect, n is the Hill constant, IC_{50} is the drug concentration producing 50% of the E_{max}, and C is the drug concentration in plasma.

Irritation or injury to the body leads to the release of arachidonic acid, which is metabolized by enzyme systems leading to the formation of inflammatory mediators termed, eicosanoids. Eicosanoids, such as prostaglandins and thromboxanes, are involved in many physiological systems, including pain, inflammation, and modulating the regional flow of blood to tissues. Cyclooxygenase is the first enzyme in the pathway leading from arachidonic acid to prostaglandins and thromboxanes. NSAIDs like flunixin and meloxicam work by blocking cyclooxygenase (COX), as depicted in Figure 8 below.

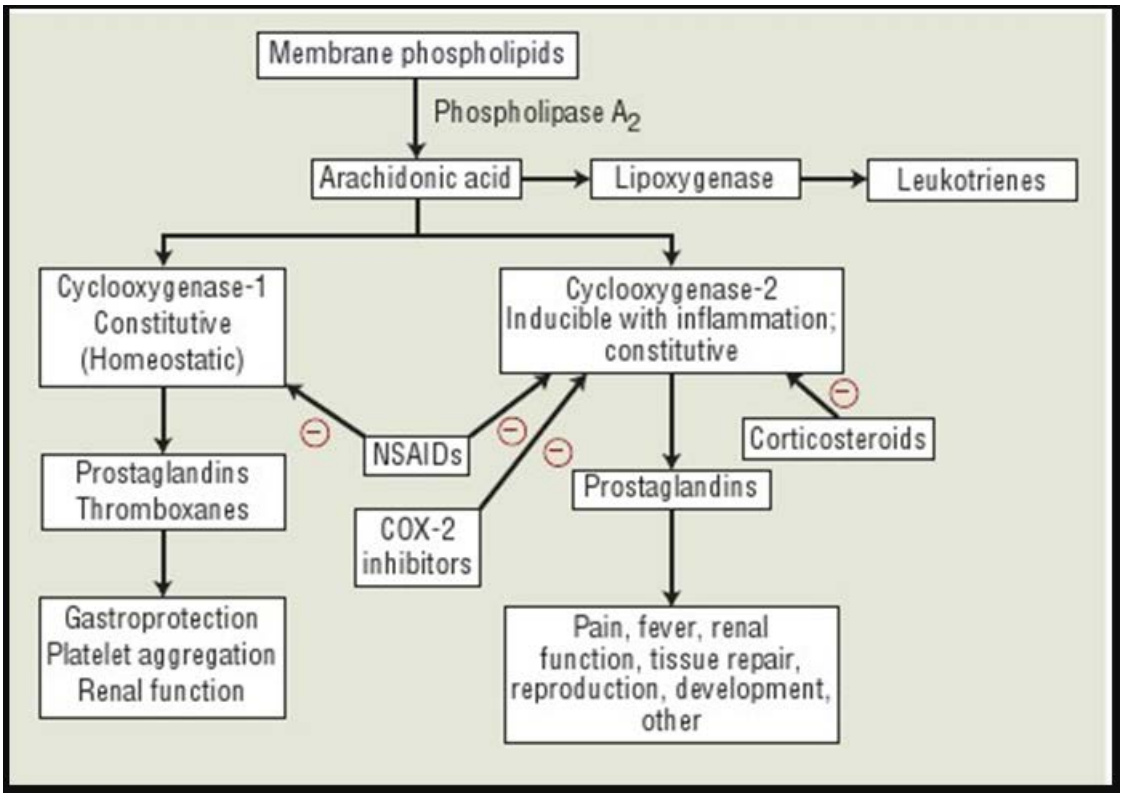

Figure 8 Inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) by nonsteroidal anti-inflammatory drugs (NSAIDs).

(https://www.memorangapp.com/flashcards/61816/2.10+-+Anti-Inflammatory+Drugs/)

The inhibiting effects of COX isoenzymes by NSAIDs has been demonstrated by measuring serum thromboxane (TXB_2) concentrations generated by platelets in clotting blood (COX-1),[39-42,81,99-105] by measuring exudate prostaglandin E_2 (PGE_2) concentrations generated by carrageenan-induced inflammation in the subcutaneous tissue-cage (COX-2),[39,41,42,81,105] and by measuring plasma PGE_2 concentrations.[99-101]

Drug concentrations in plasma, at which inhibitory effects of biomarkers from NSAIDs exposure occur were evaluated as possible decision limits for animals exhibited at livestock shows. While concentrations of flunixin and meloxicam resulting in the inhibition of PGE_2 have

been measured in exudate, it is difficult to correlate exudate and plasma concentrations. Additionally, flunixin and meloxicam concentrations resulting in the inhibition of PGE_2 have been measured in equine[100] and feline plasma,[101] but these concentrations cannot be extrapolated to other animal species. Therefore, evaluation of the Hill equation as a method for establishing decision limits for therapeutic drugs detected in animals exhibited at livestock shows was based on published TXB_2 data.

Inhibition of TXB_2 from flunixin administration has been evaluated in multiple animal species. Plasma flunixin concentrations (ng/mL) that produced 50% of the maximal effect (IC_{50}) in different animal species are presented in Table 17. IC_{50} data for inhibition of TXB_2 from meloxicam administration were not available.

Species	Dose	Route of Administration	IC_{50} (ng/mL)
Ovine	1.1 mg/kg	IV	5.04 ± 3.3
Caprine	0.3 – 60 ng/mL	*in vitro*	5.93
Bovine	2.2 mg/kg	IV	24 ± 4
Equine	0.3 – 60 ng/mL	*in vitro*	11.85
Canine	0.3 – 60 ng/mL	*in vitro*	29.62

Table 17 Effect of flunixin meglumine on serum thromboxane (TXB_2). IC_{50} = plasma drug concentration which produced 50% of the maximal effect. IV = intravenous

Establishing decision limits for therapeutic drugs in exhibition animals should take into consideration the drug concentration that produces minimal effect in the animal. For example,

the decision limit should be based on the IC_{10}, the drug concentration producing 10% of the maximal effect, as opposed to the IC_{50}. Calculation of the IC_{10} can be performed using the following equation[106]:

$$IC_F = \left(\frac{F}{100-F}\right)^{\sqrt{n}} x\ IC_{50}\ ;$$

where F is the % response, n is the Hill constant, and IC_{50} is the drug concentration producing 50% of the maximal response. The IC_{10} concentrations in sheep, goats, and cattle were calculated using the IC_{50} values presented in Table 17 and a Hill constant of 1. The IC_{10} concentrations were determined to be 0.56, 0.66, and 2.67 ng/mL for sheep, goats, and cattle, respectively. It was not possible to determine a confidence interval for the IC_{10} concentrations due to the limited data available. Therefore, a safety factor of 10 was applied to account for the variability in determining these concentrations. With a safety factor of 10, the IC_{10} concentrations were determined to be 0.056, 0.066, and 0.267 ng/mL in sheep, goats, and cattle, respectively. It is important to note that these low flunixin concentrations are near the LOD of the analytical test method. This indicates the effect of flunixin on TXB_2 likely persists after the drug is no longer detectable in plasma, as previously reported.[39]

Determination of a withdrawal interval can also be made by determining the time at which minimal drug effect occurs. For example, IT_{50}, the time at which a drug produces 50% of the maximal effect can be calculated from the sigmoidal inhibitory effect model using the Hill Equation[39]:

$$E = E_{max} - \frac{E_{max}\ x\ T^n}{T^n + {IT_{50}}^n}\ ;$$

where E is the drug effect expressed in percentage inhibition, E_{max} is the maximal inhibitory effect, n is the Hill constant, T is the time, and IT_{50} is the time at which the drug effect declines

by 50%. Subsequently, the IT_{90}, the time at which the drug effect declines by 90%, can be performed using the following equation[106]:

$$IT_F = \left(\frac{F}{100-F}\right)^{\sqrt{n}} x\ IT_{50}\ ;$$

where F is the % response, n is the Hill constant, and IT_{50} is the time at which the drug effect declines by 50%. The IT_{50} for TXB_2 inhibition following intravenous flunixin administration was reported in sheep (29 ± 1.9 hours).[39] Using the equation above, IT_{90} for TXB_2 inhibition following intravenous flunixin administration in sheep was determined to be 46.8 hours. After rounding this number to 48 hours, a safety factor of 12 hours can be applied. Therefore, an appropriate withdrawal interval to achieve an IC_{10} of TXB_2 inhibition following intravenous flunixin administration in sheep is 60 hours. IT_{50} was not reported in other species.

Advantages and Disadvantages

One advantage with the sigmoidal E_{max} model using the Hill Equation to establish decision limits for therapeutic drugs detected in animals exhibited at livestock shows is the fact that decision limits are based on pharmacodynamics. The other 2 methods do not take into consideration drug effect in the animal. This makes the sigmoidal E_{max} model using the Hill Equation a more desirable approach to establish decision limits.

One disadvantage with using the Hill equation as a model to establish decision limits for therapeutic drugs detected in animals exhibited at livestock shows is correlating IC_{50} and IC_{10} between plasma and urine. Most shows collect urine for drug testing exhibition animals such that decision limits for therapeutic drugs detected in urine based on the Hill equation must be obtained using plasma data. In the case of flunixin, inhibition of TXB_2 likely persists after the drug is no longer detectable in plasma, which creates even more of a challenge when trying to correlate plasma and urine drug concentrations.

Another limitation with the sigmoidal E_{max} model using the Hill Equation is that there are currently data gaps that will delay implementation of this approach. While IC_{50} inhibition of serum TXB_2 concentrations from flunixin administration has been measured in most food animal species, plasma PGE_2 concentrations have not. PGE_2 correlates better with the performance-enhancing effects (COX-2) flunixin has in exhibition animals than TXB_2, and is therefore a more applicable biomarker. Additional studies will need to be performed in food animal species to determine IC_{50} inhibition of plasma PGE_2 from flunixin administration. Another data gap that exists with the sigmoidal E_{max} model using the Hill Equation is lack of information for IT_{50} associated with plasma TXB_2 and plasma PGE_2 in cattle, sheep, goats, and pigs. Determination of IT_{50} is necessary to establish withdrawal intervals for plasma and urine.

Measurement Uncertainty

Establishing decision limits for therapeutic drugs detected in exhibition animals will require a change in how analytical testing is performed for livestock shows. Currently, test results are reported qualitatively (*e.g.*, positive or negative). Implementing decision limits will require the testing laboratory to perform quantitative testing, determining the concentration of the drug in the sample, and then forming a conclusion by comparing the measured concentration to the decision limit. Determining the uncertainty associated with the analytical assay and measured drug concentration will be critical in determining whether a sample is positive or negative. Uncertainty is an interval associated with a measurement result that expresses the range of values that can reasonably be attributed to the quantity being measured.[107] Uncertainty is a basic characteristic of any measurement and is always present, at every step of a procedure.[108]

Analytical testing laboratories determine measurement uncertainty during method validation. The first stage of determining the measurement uncertainty is to identify the sources

of uncertainty for the method. There are a number of possible sources of uncertainty in measurement that are not only introduced by the analytical equipment and test methods, but also by the person performing the test, data analysis, and the environment. Some of these sources include purity of reagents, sampling, measurement conditions, sample effects, computational effects, and random effects.[109,110] Random error, errors that fluctuate due to the unpredictability or uncertainty inherent in the measuring process, affect precision. Systematic error, errors associated with incorrect equipment calibration or a flawed experimental design, affect the validity of the measurements and cause bias. Once the sources of uncertainty have been identified they require evaluation, which is done by determining the ruggedness and precision of the method by performing recovery studies. Finally, once the individual uncertainty components for the method have been calculated, they must be combined to give standard and expanded uncertainties for the method as a whole.[110]

In analytical testing for drugs and chemicals, the standard uncertainty can be evaluated from the dispersion of repeated measurements and calculated using the standard deviation of the mean[111]:

$$S_{\overline{X}} = \frac{S}{\sqrt{n}};$$

where[111]:

$$S = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \overline{x})^2}{n-1}};$$

then the expanded uncertainty can be determined by multiplying the standard uncertainty by the chosen coverage factor, *k*. For most purposes, *k* is set to 2. However, if the standard uncertainty is based on statistical observations with relatively few degrees of freedom (less than about six), *k* is equal to the two-sided value of Student's *t* for the number of degrees of freedom and the level of confidence (normally 95%).[111] Finally, the result is reported as:

Result = x ± uncertainty (units).

For illustration purposes, let us assume the method validation for flunixin in urine included twenty individual negative urine aliquots spiked at a concentration of 20 ng/mL. Quantitative values for each aliquot were obtained by measuring against a calibration curve that consisted of the following calibrators spiked in urine: 5 ng/mL, 10 ng/mL, 15 ng/mL, 20 ng/mL, 25 ng/mL, and 40 ng/mL. The mean was 19.865 ng/mL and population standard deviation (S) was 9.57. The standard deviation of the mean taken from the population ($S_{\overline{X}}$) was 2.14. When multiplying by a *k* of 2 (95% confidence interval), the expanded uncertainty was 4.3 ng/mL. For illustration purposes, let us assume the threshold for flunixin in urine is 20 ng/mL. Therefore, when taking into consideration the uncertainty of the validated method, a sample concentration below 24.3 ng/mL would be considered below the decision limit.

While variability exists in measuring drug concentrations in biological samples, there are also sources of variability when assessing pharmacokinetic and pharmacodynamic parameters in animal populations. Sources of variability when performing PK/PD analyses can include breed, disease, age, gender, weight, drugs given concomitantly, and various behavioral and environmental factors.[47,112] Population pharmacokinetics is used to study how a drug varies in patients who are representative of the target population to be treated with the drug.[113,114] Population pharmacokinetics is being increasingly used in drug development to assess drug safety and efficacy, and thus is usually performed in a relatively large number of patients. Non-population pharmacokinetic studies are usually carried out in small numbers of healthy animals, which can create challenges in predicting drug disposition in animals that are sick. However, unlike population pharmacokinetics, non-population pharmacokinetics does not allow for screening and quantification of covariates for explaining variability. Therefore, it may be

necessary to apply a safety factor to account for variability that is difficult to extrapolate or measure. For example, determination of tolerances for edible tissues by the FDA Center for Veterinary Medicine involves toxicology studies that are performed to determine the acceptable daily intake (ADI) of residues in edible tissues. The ADI is based on the highest dose of a drug that produces no observable effects, which can be a no-observed-effect level (NOEL) or a no-observed-adverse-effect level (NOAEL). A safety factor, which reflects uncertainties associated with the extrapolation of data and information from toxicology studies to humans, of 100 is then applied.[54]

Regulatory Decision Making

Unlike the horse racing industry, there is no central regulatory body to create policies and provide guidance on drug testing in exhibition animals. Therefore, each show creates and enforces their own rules. This creates challenges for exhibitors as they must be familiar with and adapt to a different set of rules as they travel from show to show. This also creates challenges for veterinarians as one show may allow a certain drug to be in an animal's system during exhibition while another does not. For example, flunixin is regulated by most shows with a "zero tolerance" policy. However, some shows allow flunixin to be in an animal's system during the exhibition.

Another challenge with regard to regulating drug testing in exhibition animals is the lack of standardization in testing. Each laboratory performs drug testing using different extraction procedures, equipment, test methods, and instrument methods. Therefore, detection of drugs in exhibition animals can vary among laboratories. Some laboratories have equipment that offer remarkable sensitivity, while others use less sensitive testing methods or instruments. Testing at the limit of detection of the analytical assay can cause discrepancies in test results between laboratories. For example, it is possible for a drug to be detected in an animal by one laboratory,

but not detected in that animal by another laboratory. As a result, the test method employed must be considered when comparing test results between 2 independent laboratories from the same animal.

Establishing decision limits for therapeutic drugs detected in animals exhibited at livestock shows will create some standardization in the livestock show industry. For example, decision limits will create uniformity in how laboratories distinguish between a positive and negative sample, regardless of differences in extraction procedures, equipment, test methods, and instrument methods. Additionally, livestock shows and fairs are more likely to standardize drug testing policies with the implementation of decision limits that are based on scientific data.

Based on the data presented in Chapter II and described throughout, flunixin presents the greatest need for establishing a regulatory decision limit. However, other therapeutic drugs, such as meloxicam are used in exhibited livestock and these drugs also will require attention from livestock show regulators for establishing decision limits. Other therapeutic drugs used and detected in exhibition animals that should be considered and evaluated include phenylbutazone, dexamethasone, acetaminophen, lidocaine, isoflupredone, methylprednisolone, firocoxib, and ketoprofen. Some of these drugs are NSAIDs and potential pharmacodynamic biomarkers have been identified and described. However, a review of the scientific literature to identify potential PD biomarkers is necessary for those drugs that are not NSAIDs.

Conclusions

The 95/95 TI method is based on a withdrawal interval that is agreed upon by the regulatory body. In horseracing, determination of this withdrawal interval takes into consideration the amount of time that must pass to achieve negligible performance-enhancing effects in the animal. However, it is difficult to gauge when such an interval occurs in food

animal species, particularly when assessing drug concentrations in urine. If livestock show regulators decide plasma is a more viable sample to test for therapeutic drugs in exhibition animals, perhaps the 95/95 TI can be applied as it will be easier to determine an appropriate withdrawal interval in plasma.

The IPC/IUC method presents a challenge for livestock show regulators as it does not take into consideration performance-enhancing effects in the animal and will require the determination of a safety factor agreed upon by all livestock shows. This method also has limitations in that it cannot be used for drugs whose effects last long after the drug has been eliminated.

Using the Hill equation to establish decision limits for therapeutic drugs detected in exhibition animals provides a logical approach as it accounts for drug effect in the animal. Application of this method for flunixin as described above was solely based on inhibition of TXB_2. However, inhibition of PGE_2 also occurs and thus additional studies are warranted in food animal species to determine IC_{50} for PGE_2 inhibition prior to making regulatory decision limits. As noted above, IC_{10} values for TXB_2 inhibition from flunixin administration were close to the LOD of the analytical test method. This suggests that the inhibitory effects of flunixin on TXB_2 likely persist after the drug is no longer detected in plasma. Thus, “zero tolerance” may be a valid argument for flunixin detected in plasma. While physiologic inhibition of TXB_2 and PGE_2 occur at the IC_{10}, it is unclear if these inhibitory effects result in a performance-altering effect in the animal that creates an unfair advantage during competition.

While one or more of the methods described for establishing decision limits for therapeutic drugs detected in animals exhibited at livestock shows may provide a path forward, additional data are needed. First, pharmacodynamics using biomarkers allows the measurement

of drug effect in the animal. However, it is unclear if detection of these biomarkers at any level correlates with a performance-altering effect in an exhibition animal. Also, PGE_2 appears to correlate more strongly with the desired performance-enhancing effects of NSAIDs in exhibition animals than TXB_2. Therefore, studies measuring plasma PGE_2 levels following NSAID administration in cattle, sheep, goats, and pigs are warranted. Evaluation of other biomarkers of NSAID exposure for potential implications on altered performance is also recommended. Second, it is difficult to correlate drug concentrations in plasma and urine from information published in cattle, sheep, goats, and pigs. Data describing detection of drugs in urine over time in these species are limited. PK studies, similar to those performed in Chapters III and IV, in other animal species are necessary to obtain information on drug disposition in both plasma and urine, and to evaluate their correlation. This is especially true if livestock shows are to continue regulating doping in exhibition animals by testing urine. Last, establishing decision limits for therapeutic drugs detected in exhibition animals should account for pharmacokinetics and pharmacodynamics of animals with disease. Exhibition animals requiring treatment for an illness prior to a show may have differences in drug disposition and elimination compared to healthy animals. PD biomarkers may also behave differently in animals with disease as opposed to healthy animals.

CHAPTER VI

CONCLUSIONS

Conclusions

Improvements in analytical test methods in recent years have called into question the appropriateness of current regulation of drug testing in animals exhibited at livestock shows using a so-called “zero tolerance” approach. While this policy is appropriate for prohibited substances, detection of trace amounts of therapeutic drugs, such as flunixin, used in a legal manner has raised concerns within the livestock show industry. First, detection of trace amounts of therapeutic drugs in urine raises the question of whether or not performance-enhancing effects occur in the animal at these very low concentrations. Second, veterinarians face challenges when providing care to exhibition animals, as they are unsure of appropriate withdrawal times for therapeutic drugs in urine to avoid an adverse analytical finding from unintended consequences. Last, the possibility of environmental contamination from the co-mingling of treated and untreated animals has been demonstrated.[8,23] Thus, the need to establish decision limits for therapeutic drugs detected in animals exhibited at livestock shows is evident.

PK studies, like those performed in Chapters III and IV, are necessary to evaluate the movement of a drug into, throughout and out of the body; that is, the time-course of the drug’s absorption, bioavailability, distribution, metabolism, and excretion.[32] Likewise, this information is also important when establishing decision limits. Most livestock shows collect urine from exhibition animals for drug testing. However, the flunixin PK data presented in Chapter III provides an example of the variability of drug concentrations in urine. Urine provides a longer detection time for some drugs compared to plasma. While this is advantageous for prohibited

substances, this may not be the case for therapeutic drugs. Duration of therapeutic effects correlates better with plasma drug concentrations.[43-46,81,82] Thus, consideration should be given to regulating the use of therapeutic drugs, like flunixin, in animals exhibited at livestock shows using plasma as opposed to urine.

Three different methods for establishing decision limits for therapeutic drugs detected in animals exhibited at livestock shows have been described. The first method, the 95/95 Tolerance Interval, is based on a withdrawal interval that is agreed upon by the regulatory body. Determination of this withdrawal interval should take into consideration the amount of time that must pass to achieve negligible performance-enhancing effects in the animal. This presents a challenge for livestock show regulators as it is difficult to gauge when such an interval occurs in food animal species, particularly when assessing drug concentrations in urine. The second method, the irrelevant plasma concentration (IPC) and irrelevant urine concentration (IUC) is based on the standard drug dose and plasma clearance to determine the effective plasma concentration. Then the IPC is determined by applying a safety factor, which again is a regulatory decision. Last, the IUC is obtained by multiplying the IPC by the steady-state urine to plasma concentration ratio. The IPC/IUC method presents a challenge for livestock show regulators as it does not take into consideration performance-enhancing effects in the animal and will require the determination of a standardized safety factor. The third method described for establishing decision limits for therapeutic drugs detected in animals exhibited at livestock shows was the sigmoidal E_{max} model using the Hill equation to determine the IC_{10}, the drug concentration producing 10% of the maximal effect. This method also allows for the determination of the IT_{90}, the time at which the drug effect declines by 90%. While this method takes into consideration physiologic effects produced by a drug, it was not possible to apply

limits derived from plasma data to data from urine, which is all that is available for exhibited livestock.

Should livestock show regulators decide to test for therapeutic drugs in plasma rather than urine, the author recommends establishing decision limits that combine 2 of the methods described in Chapter V. First, the drug concentration that produces minimal effect in the animal should be determined (*e.g.*, IC_{10}). Then the time to reach this concentration (*e.g.*, IT_{90}) should be calculated. Finally, the 95/95 TI method can be performed using this withdrawal interval to determine the regulatory threshold and subsequent decision limit.

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