2021

SCIENCE-BASED REGULATION OF PHARMACOLOGICAL SUBSTANCES IN COMPETITION HORSES

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Digital Object Identifier: https://doi.org/10.13023/etd.2021.402

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SCIENCE-BASED REGULATION OF PHARMACOLOGICAL SUBSTANCES IN COMPETITION HORSES

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By

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2021

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ABSTRACT OF DISSERTATION

SCIENCE-BASED REGULATION OF PHARMACOLOGICAL SUBSTANCES IN COMPETITION HORSES

Current testing methodologies within equine forensic toxicology focus on arbitrary thresholds and zero-tolerance policy. Modern analytical chemistry's limits of detection are low enough that oftentimes femtogram-per-milliliter amounts of a substance can readily be identified in both blood and urine of a horse. For most pharmacologically relevant compounds, these concentrations have no relevance to pharmacological effect. It is therefore crucial that testing methodologies to determine appropriate thresholds and cut-offs be developed that are driven by biological activity rather than arbitrary limits of detection. This dissertation looks to address this by suggesting a system of calculated Effective Plasma Concentrations by which a safety factor may be determined to calculate an Irrelevant Plasma Concentration that may act as a regulatory threshold. In support of such pharmacokinetic studies, novel synthetic pathways have been developed to produce reference standards and stably isotopically labelled deuterated internal standards.

The research undertaken in this thesis is comprised of three primary areas of focus: (1) the development of novel synthetic pathways for certified reference standards and internal reference standards, (2) the pharmacokinetic description of biologically relevant compounds using modern techniques so as to guide both horsepersons and regulators in the horse industry, and (3) the description of environmental contamination risk for compounds and their ability to be taken up by the animals and later detected by forensic investigators from these residual exposures.

Of the first category, three papers are presented, each representing the novel synthesis of a different compound of biological importance. The first compound synthesized was deuterated (d_6) xylazine as use as an internal standard for quantitation of the therapeutic compound xylazine. The second compound was the novel synthesis and purification of barbarin, a naturally occurring glucosinolate which is believed to be responsible for the appearance of a DEA Schedule I compound in horse urine. The

synthesis of barbarin as described is the first known chemical synthesis of the compound, which until this point had only been previously extracted from biological samples. The third paper covers the synthesis of a deuterated (d_5) barbarin for use as an internal standard in its quantitation.

The second category covers the pharmacokinetic studies, the first of which is a pilot study which looked at the terminal half-life of mepivacaine in thoroughbred horses. This study offers guidance for horsepersons and veterinarians on proper withdrawal times for the given regulatory thresholds of the compound when using therapeutic doses up to four-hundred milligrams as well as describing the pharmacokinetic model developed in determining these withdrawal times. The next study looks at methylprednisolone administration across multiple joints and the effects of specific joint injections and co-injections on the detection of the compound out to six days post-administration. This paper also offers guidance to withdrawal of animals after therapy and highlights many of the problems in current regulatory guidance. The final paper looks at betamethasone in harness racing and suggests testing thresholds based upon pharmacokinetic and statistical models at a six-day withdrawal timeline.

The final paper discusses the likelihood of the commonly used non-steroidal anti-inflammatory drug naproxen to contaminate the environment of the horse. It also looks establishes a suggested screening limit of detection for the compound based upon the concentrations of a series of innocuous positives that appeared at a racetrack upon changing testing facilities. This paper highlights the importance of consistency between testing facilities as well as the need for expanded established limits instead of the zero-tolerance regulation of most compounds.

Finally, the dissertation concludes with a synopsis of the research presented along with suggestions on how the racing industry may move forward in its testing of the animals in both a way that will be less burdensome in the future as well as more appropriate to protecting the health and livelihood of the animals on which it relies.

KEYWORDS: Equine Forensic Toxicology, Detection Limits, Drug Testing, Regulatory Models, Novel Syntheses, Environmental Contamination

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SCIENCE-BASED REGULATION OF PHARMACOLOGICAL SUBSTANCES IN COMPETITION HORSES

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DEDICATION

To my mother Catharine Schmitt and my father William Machin, both of whom I could not have made it this far in life without their daily inspiration, both moral and intellectual.

ACKNOWLEDGMENTS

Throughout the writing of my dissertation and the near endless research I received a great deal of support and assistance.

I would first like to thank my supervisor, Dr. Thomas Tobin, whose expertise and insight into the field of Equine Forensic Toxicology was invaluable in illuminating the need for better protections for the health of these animals that will also protect those who care for them. His thoughtful and caring approach has improved my capabilities as a scientist as well as a caretaker of my fellow man and animals.

I would also like to acknowledge Dr. Clara Fenger, with whom we regularly collaborated and for her fierce dedication to proper medical care for these wonderful animals. She inspired me to find a focus in life with which to dedicate myself and not take no for an answer.

Finally, I would like to thank my family, both blood relatives and those who I share my love with, without their support I could never have made it this far in my journey. They have built me up when I felt that I was doomed to fail.

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CHAPTER 1. INTRODUCTION

1.1 The World is Watching

Equine Forensic science made worldwide news at the 1968 Kentucky Derby. The Sunday before the world-renowned race, the horse Dancer's Image was reportedly treated with phenylbutazone, a common nonsteroidal anti-inflammatory drug [NSAID]. The following Saturday, Dancer's Image would go on to win the Kentucky Derby, only to be later disqualified due to testing positive for phenylbutazone (Hackett, 1968). The controversy made world-wide news, including the cover of *Sports Illustrated*, and brought equine forensics to the forefront of many debates. Current rulings now allow a threshold amount of phenylbutazone to be detected in the horse at competition – 0.3 micrograms per milliliter of plasma or serum – and these levels are expected to be reached by 48 hours of the administration of a single dose (RMTC 2019). However, in 1968, quantitation of the substance was not possible, so any 'positive' was disqualifying.

The 'positive' was contested by the owner, Peter D. Fuller. Theories began to crop up with those that believed Fuller, including the likelihood of a malicious dosing of the therapeutic compound the day of the racetrack in order to cause a 'positive' result to appear (Cady, 1970). While this may sound far-fetched, Fuller had made enemies in the industry and the region, as he supported the civil rights movement, including donating the winnings from a previous race to Coretta Scott King two days after the assassination of her husband earlier that year.

Only six years later, in 1974, phenylbutazone would be legalized for use in horses by the Kentucky Racing Commission, however to this day, Dancer's Image is officially considered a disqualification (Christine, 1988). With such high stakes on the line, how then are stewards of Thoroughbred racing best posed to protect not only the health of their horses, but the image of the industry as a fair and balanced community? Modern testing now allows for detection of most therapeutic substances to a much more extreme level and understanding the need for regulatory thresholds will only help prevent another such scandal from surfacing.

1.2 The World is Watching, Again

Until May 9, 2021, no other horse to cross the finish line at the Kentucky Derby tested positive for a substance above the allowed substance limit. However, international attention was brought upon the race once again as the horse to finish first, Medina Spirit, would go on to test positive for the glucocorticoid betamethasone at a reported level of 21 picograms-per-milliliter of blood. The Association of Racing Commissioners International [ARCI] and Racing Medication and Testing Consortium [RMTC] at the time recommended a threshold limit of 10 picograms-per-milliliter in blood for betamethasone in harness races and zero-tolerance in flats races, lowered from its previous recommended threshold of 100 picograms-per-milliliter in blood with no citation as to the reason presented by the RMTC. The Kentucky Horse-Racing Commission [KHRC] had recently changed the policy regarding the pain reliever to a zero-tolerance limitation for detection at the time of the race (Paulick Report Staff, 2021).

Media attention soon swamped the public with reports of a 'banned steroid' that would disqualify Medina Spirit's claim to being the Kentucky Derby winner (Managan 2021). The world soon came crashing down on the horse-racing industry, as personalities ranging from athletes, animal rights' activists, and more made commentary on the use of steroids on animals. This effect served no positive purpose to the horseracing industry besides to tarnish its reputation. Betamethasone, while technically a steroid, is best described as a glucocorticoid and is predominantly used to help with joint pain. Even if levels detected in Medina Spirit were from those introduced to the correct physiological structure rather than topically, the reported levels would almost certainly offer no pharmacological relevance to the race in question, in my opinion. Possibly more importantly to the public image of horseracing, however, is the fact that betamethasone is not banned from the industry and is commonly used to help prevent inflammation and immune response in joints that are worked hard during training. While it is regulated at the horse track, at the levels reported, it is far from the performance-enhancing drug [PED] as the media painted it but is instead a legitimate therapeutic that is properly used to maintain the health and welfare of the animal.

The trainer of Medina Spirit, Bob Baffert, would contest the presence of betamethasone and request a split sample analysis for confirmation. Initially, Baffert claimed that they had not administered betamethasone to the animal whatsoever and suggested that it was perhaps a malicious administration. This statement would later be reversed when it came to his attention that a topical ointment, Otomax, that had been used to treat topical dermatitis Medina Spirit had developed on her hindquarter. Otomax's primary therapeutic compound is gentamicin sulfate, a broad-spectrum anti-yeast and anti-bacterial compound used to control otitis in canines. Betamethasone is present in the ointment as well to help treat inflammation at the site. Clotrimazole is an antifungal present in the ointment as well. Baffert claims that the ongoing treatment of Medina Spirit with Otomax was likely the cause of the presence of betamethasone in the animal at the time of the race and holds that it had no effect on the integrity of the race itself (Frakes, 2021).

On June 2, 2021, the split sample results were announced, indicating that 25 picograms-per-milliliter of betamethasone had been detected in the blood of Medina Spirit. No further testing has been announced at the time of this writing, though Clark Brewster, the attorney representing Baffert, has stated that they plan on testing the samples for other compounds in the Otomax ointment to further strengthen their case that the presence of betamethasone was due to the use of said ointment rather than a joint injection (Bonesteel, 2021). Meanwhile, arguments are being prepared by both parties involved on whether that such positives should be considered rules violations or be shown leniency. Regardless of the outcome, the world will more likely remember the 'drug use in horseracing' headlines, and count this as a strike against the integrity of the sport.

1.3 Financial Impact of the Industry

As of a 2017 study, the United States horse industry contributes fifty billion dollars in direct economic impact to the U.S. economy and supports approximately one million jobs. Including indirect and induced spending, this impact totals over about 122 billion dollars and a combined effect on employment of 1.7 million jobs. Undoubtedly, these numbers have since increased since 2017. Of the 7 million horses in the United States,

approximately 1.2 million are active in horseracing, which comprises one of the largest economic impact sectors of the US equine industry (AHC, 2017).

Over 165 million dollars in wagers were placed on the 2019 Kentucky Derby alone (Paulick Report, 2019). As an indicator for money both generated by the industry and wagered within the industry, a focus on the fairness of the sports medicine applied to the athletes (the horses) is of utmost importance, both in terms of the protection of the bettor and, more importantly, in terms of the protection of the animals themselves. The key discipline in offering regulators a window into the pharmacological mechanisms active within the horse at the time of the race, and therefore the leading technology for protecting these animals, is equine forensic science.

1.3.1 Equine Forensic Science

Current Equine forensic toxicological practice within the United States for the sports horse industry focuses on a zero-tolerance regulatory concept, with defined exceptions for specific therapeutic medications, and endogenous, dietary, and environmental substances. That is, allowable levels of therapeutic compounds are those which are below the analytical level of detection, unless a specific threshold is established. However, as of the start of 2020, this extends only to twenty-eight Controlled Therapeutic Medications, three of which are Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), which are restricted to a single type being present per forensic sample (RMTC, 2020).

The thresholds established for the above classes of medications range from 3 picograms per milliliter (3 parts per trillion) in plasma for glycopyrrolate, an anticholinergic, to 10.0 micrograms per milliliter (10.0 parts per million) in plasma for dimethyl sulfoxide [DMSO], a topical analgesic and transdermal carrier. Most compounds, however, are regulated in the range of picograms (9 total) to nanograms (17 total) per milliliter.

The Association of Racing Commissioners International [ARCI] publishes these thresholds and cites the “dosing specifications” from which these thresholds are established but does not always provide a direct citation of the studies on which they are based, though

a reference chart is offered for some example studies (ARCI 2020; RMTC, 2017). However, they do provide for horsepersons a suggested "withdrawal guideline" based on these administrations that represent guidance for their estimated time for the experimental administration dosage to reach a level that should be below the established threshold, as calculated by a "95/95 Tolerance Interval" (RMTC, 2016).

A 95/95 Tolerance Interval is a statistical interval which predicts the values by which 95% of a given population will fall within a range (at 95% confidence). That is, given 100 members of the population, i.e., racehorses, it is assumed that there is a 95% chance that 95 of those horses would be predicted to show behavior, i.e., test below the given range/threshold. Within the RMTC case, this is taken as a one-sided interval (that is, 95% of the population will fall at or below the threshold). The guidelines put forth by the RMTC also assume that the any residual amounts within the population will follow normal (or log-normal) distributions, which is often not statistically proven for any given regulated compound. While this is the case for many biological functions, it fails to take account of many confounding sources of variance, such as the potential presence of genetically distinct drug metabolizing subpopulations or differences in exercise, diet, gender, or other environmental factors.

On June 12, 2021, Pimlico Racecourse ran eight races, totaling 84 different thoroughbreds that ran through the starting gates that day. The mathematical assumption that 5% of those that received legitimate therapeutic medications that end up being tested may be disqualified from the race points to an inevitability – the penalizing of innocent trainers, owners, and their animals for the presence of a substance that had no effect on the integrity of the race itself. As long as thresholds continue to be based on withdrawal windows that are arbitrarily determined rather than on physiologically relevant concentrations, this will continue to be a major issue for the health of the animals.

These realities present several significant challenges and obstacles for horsepersons. Even under ideal circumstances and following these guidelines rigorously, it would be predicted that approximately one out of every twenty animals that were responsibly treated while following the withdrawal guidelines would potentially be flagged as over the limit ('called'/'identified' as described later). While that may seem

inconsequential, these horses are athletes and as such should have readily available access to certain pharmaceuticals that improve their quality of life when they have no effect on the outcome of a competition. Zero-tolerance policies, however, discourage horsepersons from administering these therapeutics when they are in good recommendation but not absolutely required. These penalties are assessed against the owners and trainers of these horses and the severity of such penalties is only determined by the number of positive tests that trainer has received in his recent past (generally a one-year window). This means that a successful trainer that fields one-hundred different racehorses and has two positive tests over the course of the year is systemically considered an equally than a trainer that fields a single horse and tests positive the same number of times.

However, it is rare that the ideal circumstances will be met for a therapeutic, and so many pharmaceuticals' pharmacokinetics may be reported based on inappropriate or outdated studies. While subpopulations are one confounding variable, the increased detection capabilities of a modern mass spectrometer have allowed for a better understanding of 'terminal' half-lives, which for most compounds appear to be much longer than previously reported. This decreased rate at which the pharmaceuticals are cleared from the body at low concentrations increases the potential for an innocent 'positive.' Guidance from commissions often assumes previous 'terminal' half-lives are the truly terminal (rather than the half-life that has been measured at the time of sampling), which inevitably leads to the innocuous presence of legitimate therapeutics as detection levels become increasingly sensitive – leaving the horsepersons penalized for being good caretakers of their horses. This circumstance is a major problem for the industry as a whole, and understanding it requires understanding the regulatory processes used by most commissions.

1.4 The Regulatory Process

"How are these medication regulations implemented?" one is left asking. Modern equine forensic toxicology is primarily governed by state commissions which set the rules (in the United States, often in line with those suggested by the RMTC/ARCI). However, testing itself is generally carried out by independent laboratories. These laboratories test

blood and urine samples for therapeutic compounds via an array of methodologies and generally confirm their identifications/quantitation via mass spectrometry. Whenever a regulated substance is found to be above the defined threshold it is their task to report these findings to the relevant commission, which may then proceed with regulatory action against the trainer/owner.

There are several terms that should be carefully defined for this discussion. Oftentimes you will hear the term 'positive' used to describe the detection of a pharmaceutical or other bioactive compound within a testing environment. Within the context of the racing industry, it is important to differentiate between 'identifications,' 'calls,' and a 'positives.' An 'identification' in this case is the forensic laboratories identifying the presence of a compound of interest in the test samples. A 'call' is whenever said compound is reported to the commissions for being present above a threshold. Finally, a 'positive' is the step when a commission considers a rules violation to have occurred and undertakes proceedings against person for violation of rules. Note that this may differ slightly from the ARCI definition of a 'positive' which states that a 'Positive Test' "is a finding certified by the Laboratory that a regulatory analyte from a Prohibited Substance is present in the sample in an amount that exceeds the regulatory limit" (ARCI, 2019) However, in practice these will often be one and the same, as commissions rarely, if ever, decline to pursue penalties against trainers whenever a substance is reported – that is, while our definition of a 'call' may be closer to the ARCI's definition of a 'Positive Test,' a 'call' will generally lead to a 'positive.'

1.4.1 In-House Testing Thresholds

However, a complication in the overall testing process is that some testing facilities and jurisdictions have established their own 'in-house' thresholds for certain compounds that are commonplace – such as naproxen. These values are not available publicly but serve as a means to bypass lengthy legal proceedings and technical questions.

These in-house thresholds lead to many downstream problems, however. The lack of clarity on what is being reported is a prime source of confusion. Naproxen, for example, has no listed RMTC threshold, and so is supposedly a zero-tolerance offense. This means that while many commissions have a ruling on paper of 'zero-tolerance' for the compound,

they in practice have a threshold, based upon their testing facilities discretion. This value is rarely known to either the horsepersons or commissioners, and so practical guidance is impossible for recommended dosing and withdrawal. However, the regulators often communicate what is viewed as an effective withdrawal guideline to the community, but without defined regulatory or scientific support. Likewise, any 'positive' that is called by a laboratory with a lower in-house threshold than another can create regulatory problems, depending on the concentration reported.

Just such a problem arose whenever an authority (West Virginia Racing Commission) within the United States changed testing facilities from one (Truesdail Laboratories) with an in-house testing limit for naproxen to one without (Industrial Laboratories). Suddenly, low-level positives for the compound began being called in the region for horsepersons that previously had a clean record of any violation. Further investigation revealed that the change in testing facilities accompanied an alteration to a zero-tolerance in-house threshold for the compound. Statistical work on the low-level reports indicated that an appropriate interim threshold of 250 nanograms-per-milliliter of urine seemed appropriate. While this recommendation seems to have been effective for the time being, the compound still does not have a recommended threshold by the RMTC/ARCI. However, without an up-to-date pharmacokinetic model, it is unlikely that a meaningful withdrawal guideline can be given to the industry.

Despite the recommendations and guidelines put forth, sporadic 'positives' will often still occur from members of the racing community with no known history of medication abuse that leave both the individual and commissions confounded by their origin. Classic examples include apparent human-to-horse transfers for tramadol, dextromethorphan, and cocaine/benzoylecgonine [BZE]. These positives often imply one or multiple issues with the current regulatory methodologies, as the source of the medication is innocent and inadvertent, while the levels reported are often physiologically irrelevant. In other cases, trace contaminants in the environment may lead to the identification of a compound in the forensic sample, either by introduction during or after collection or inadvertent exposure to the athlete. This second point is especially problematic for compounds that are ubiquitously used by humans and either

environmentally stable or particularly readily detected (cocaine, caffeine/chocolate, naproxen).

These sporadic, environmentally-related 'positives' should be of use to regulators when appropriately understood, however. If they occur in large enough numbers with no known cause, it is often possible to determine a specific reasonable cut-off threshold. For example, a published paper discussed in more detail later showed an appropriate threshold of 250 nanogram-per-milliliter urine for naproxen used outlier analysis to determine an interim threshold (Machin, et al 2020). This not only helps in the absence of studies using modern detectors, but also informs the presence of environmental sources and what may be considered unobjectionable exposures. This emphasizes on the need to have 'in-house' testing thresholds reported so that they may be reviewed. Meta-analyses of previous studies may also offer similar insight, as they allow aggregation of datasets to better understand the pharmacology of the population at large.

'In-house' thresholds are not the only problem with lack of clarity presented by the methods of regulation in modern equine forensic toxicology: many of the thresholds in place cite literature which is either not specified, unpublished, or failed to be cited all together. Even when the citations are publicly available, the interpretation of the data is not, resulting in ambiguity with regards to exact methodology used in determination of these thresholds and withdrawal timelines.

Horsepersons often must simply take these rulings as presented by the authority that the rules and regulations are not only being formulated appropriately but being enforced in a sensible way, as well. Without access to the studies and interpretations of those making the rules, updates to the system are unlikely to occur with haste or accuracy.

This is especially problematic for legitimate therapeutics that are not listed by the RMTC/ARCI, indicating that their allowable levels fall below that of the Limit of Detection of a modern mass spectrometer. It also, however, is a problem for illicit substances that may be found in the environment of the athletes and their staff.

Ultimately, the application of zero-tolerance detection policies will only cause further headaches in the future as detection capabilities increase while the biology of the animals remains relatively constant. Ideally, appropriate thresholds for all compounds will be determined based on biological activity rather than simple detection. In the meantime, however, pharmacokinetic studies are of crucial importance in protecting both the animal-athletes and their caretakers.

1.4.2 In the Interests of the Horse

Ultimately, the welfare of the animals would best be served by a governing system of rules that allows for the appropriate and legitimate use of therapeutics while restricting them from competing while under the effect of such compounds. Such a system could allow for many therapeutics to be at modern detectable levels while disallowing such levels that may affect the outcome of a competition. For instance, if a High No Effect Dose [HNED] were to be determined for a compound, then some safety factor below this dose could be taken into account when establishing a relevant cut-off for detection. Not only would this system allow for legitimate therapeutic uses that protect the integrity of the competition, but it would also act to future-proof many forensic regulations, in that increased sensitivity in testing need not be considered for an established threshold. Certain compounds may still receive a "zero tolerance" ruling, such as the use of anabolic steroids, but these appear much more straight forward in citing as disallowed than the current regulatory state in which only those cited are allowed.

In fact, such a model has been proposed and is addressed and utilized in our studies on naproxen (Machin, et al 2020). While the safety factor appears to be relatively high in this case, using it as a basis from which to expand seems a well-reasoned place to start. Appropriate safety factors grounded in current thresholds for differing classes of RMTC allowed thresholds may be calculated to determine what is an appropriate interim threshold before full HNED, pharmacological, and pharmacokinetic studies may be performed. For these studies to be accomplished, appropriate reference standards need to be synthesized.

1.4.3 The Need for Reference Standards

Of critical importance in performing these pharmacological studies is access to high-quality reference standards. While many pharmacological compounds are readily available for use in standards, oftentimes their stable-isotopically labelled counterparts are not. Even more of a problem, oftentimes the reference compound of interest to the regulator is not the parent drug, but a metabolite of said compound that is not available whatsoever for purchasing as pure standards. The novel syntheses of pharmacologically relevant compounds and their labelled forms is an important and often overlooked aspect of the forensic toxicologist.

Horseracing laboratories are generally accredited under the International Laboratory Accreditation Cooperation section G7 [ILAC G7]. Under these guidelines, identification of a prohibited substance must "normally result from direct comparison with a reference material analyzed in parallel or series with the test sample." Generally, these reference materials should be either a direct chemical reference or a stable isotopically labelled form, as required for identification or quantitation, respectively.

With this in mind, the novel syntheses of many of these small molecular compounds is of great import to the regulatory systems currently in place. Both for the parent compounds of pharmaceuticals as well as their metabolites. While it is often the case that in the equine model the molecule is both hydroxylated and then glucuronidated and excreted into the urine via this secondary step of metabolism, the parent compound is often recovered via cleaving by beta-glucuronidase and hence the regulatory chemical of interest. For pharmaceutical compounds these may or may not always be available as ISO-certified reference standards, and so the small-scale synthesis is important to establish. Likewise, the development of this type of novel synthesis often opens the door to the synthesis of isotopically-labelled forms – generally via deuterium.

This is just such the case for the novel syntheses developed for xylazine and the deuterated d6-xylazine. Xylazine is an agonist at the alpha-2 class of adrenergic receptors generally used for sedation and anesthesia. It is currently suggested to have a forty-eight-

hour withdrawal period for a dose of two-hundred milligrams via IV bolus at two hundred picograms-per-milliliter in blood plasma or serum. Prior to our development, there was not a reported synthetic pathway to make deuterium-labelled xylazine for use as an internal standard, and so quantitation was limited. While only an incremental step, it is a necessary one for proper regulation of xenobiotics accepted to have thresholds.

Naturally occurring compounds are often a source of possible 'positives' as well. One unexpected culprit is the compound barbarin, a secondary metabolite and glucosinolate found predominately in its namesake plant *Barbarea vulgaris*, commonly Yellow Rocket. *B. vulgaris* is endemic to most of the northern hemisphere, and while unlikely to be eaten as a food source on its own, evidence has shown that it is readily eaten when mixed with feed and that doing so leads to the detection of aminorex in the urine of the horse afterwards (Maylin, 2019). Barbarin itself is not a regulated compound but is believed to be the causative agent in the metabolism of *B. vulgaris* in causing the detection of aminorex – a DEA Schedule I substance -- in equine urine.

Traditionally, barbarin was produced by lysing the cells of *B. vulgaris* and purifying the molecule. While relatively straightforward, it was unable to be produced in large amounts economically. With that in mind, we developed a novel synthesis of both reference standard barbarin as well as the deuterium-labeled equivalent for use as certified reference standards.

Ultimately, having a systematic approach to the development of thresholds for every class of pharmacological compounds not only would streamline regulation, but give guidance and coverage to horsepersons and veterinarians caring for their animals. As it is, the 'zero tolerance with exceptions' is quite the opposite of the ideal situation for performance horses and their health.

1.5 Pharmacokinetic Studies

With proper pharmacokinetic studies, the availability of certified reference standards, and an understanding of environmental exposures, the current system of regulation can be

greatly improved as governing bodies move towards a biology-based regulatory model. In the transition, however, a model system needs to be proposed, and that is what follows by example.

Naproxen, as described previously, is a commonly used NSAID that has a long half-life in the environment and can be readily taken back up by the equine patients that remain in the treatment environment, or those areas contaminated by other treated animals. While levels from environmental exposure are unlikely to ever be close to those that are biologically relevant, it is important that an appropriate threshold be established for its regulation. A biology-based regulatory model should take into account the 'Effective Plasma Concentration' [EPC] as described by Toutain (Toutain, 2002a, b) and some safety factor to determine what is considered an 'Irrelevant Plasma Concentration' [IPC]. This IPC should be based upon a hierarchy of knowledge for the compound.

The best situation would be a safety factor that is based on the known pharmacology of the compound; however, this is not always practical. When in-depth data is not available, looking at the class of the therapeutic and safety factors used for these should be used as guidance. Finally, when neither of these datapoints are known, an extremely cautious safety factor should be taken.

Toutain suggested an initial safety factor of approximately 500 to be conservative. While this certainly would be an IPC, it is likely too conservative to be universally applied. Our work with naproxen (Machin 2020) suggested a factor of approximately fifty, as this is within the standard range of actual regulatory thresholds for other NSAIDs. Our suggested threshold also fits well with the data available for the sporadic 'calls' that were caused by the shifting of testing facilities, so would likely cover most innocent identifications.

Certain compounds, of course, should be regulated even more strongly than a standard 'conservative' IPC safety factor. An example of this currently is guaiphenesin – an expectorant and skeletal muscle relaxant, which is approximately 1000 times less than the effective plasma concentration (or half that of Toutain's recommended conservative IPC).

Following are specific examples of related reports and research accomplished with a brief summary.

CHAPTER 2. STUDY SYNOPSES AND PUBLICATIONS

The following will consist of a cover page giving a synopsis of a study performed followed by the publication, both accepted and submitted. These articles will be broken into three primary categories: Novel Syntheses of Standards, Trace Level Therapeutic Medication Overages/Positives, and Environmental Sources. The general format will be as follows:

Article title

Abstract

Experimental (excluded in review articles)

Conclusion

Citation

Personal Contribution

2.1 Novel Syntheses of Standards

2.1.1 Synthesis, Purification, and Certification of Xylazine-d6 for Equine Medication Regulation

Abstract: Xylazine, N-(2,6-Dimethylphenyl)-5,6-dihydro-4H-1,3-thiazin-2-amine, is a short-acting alpha-2 agonist tranquilizer approved for therapeutic use in horses. Xylazine is also an Association of Racing Commissioners International [ARCI] Controlled Therapeutic Medication [CTM] with potential to affect equine performance. As such, in the United States its use in competition horses is regulated via a regulatory threshold in plasma for parent xylazine. We have therefore synthesized, purified and certified xylazine-d6, a deuterated analogue of xylazine, for use as an internal standard in quantification of xylazine. Xylazine-d6 was prepared commencing with commercially available 2,6-dimethyl-d6-aniline, which was reacted with thiophosgene to yield the isothiocyanate intermediate. Reaction of the isothiocyanate intermediate with 3-amino-1-propanol, followed by refluxing in concentrated HCl produced xylazine-d6 in moderate yield (40%) which was then purified and fully characterized by NMR, HPLC, GCMS and high-resolution mass spectroscopy for reference standard certification.

Introduction: Xylazine is a rapidly acting alpha-2 agonist and tranquilizer commonly used in the horse-racing industry. Regulatory thresholds within the US for xylazine restricted it to no more than 200 pg/mL in blood at the time of the race. Prior to this value, urinary concentrations were used to determine xylazine and used its primary metabolite (4-hydroxyxylazine. The changing to parent xylazine as the molecule of interest demands the need for a deuterated internal standard and reference standard.

Experimental:

The first reaction takes the above produced aniline and dissolves it in dichloromethane followed by the drop-wise addition of triethylamine and thiophosgene under ice-cooled conditions. The mixture is allowed to stir for an hour at room temperature

before being poured over ice and extracted into dichloromethane resulting in 2,6-d6-dimethylphenylisothiocyanate.

The final step of the preparation is dissolving the produced isothiocyanate in dichloromethane with the addition of 3-aminopropanol and allowing to reflux overnight with stirring. Solvent is removed via rotary evaporation and an addition of concentrated hydrochloric acid before being allowed to reflux overnight once again. This final solution is then poured over a 10% sodium hydroxide solution and stirred for three hours, the xylazine-d6 precipitating out as the hydrochloride salt.

The reaction and products were characterized via proton nuclear magnetic resonance spectroscopy as well as both GCMS and LCMS. Combustion analysis was used to verify composition and purity. Certificates of analysis are available for the resultant product.

Conclusion: Xylazine-d6 is now available for use in gram quantities and well characterized for use as an internal standard in forensic sciences in compliance with ISO analytical standards.

Citation: Machin, J., Kudrimoti, S., Brewer, K., Eisenberg, R., & Tobin, T. (2016). Synthesis, Purification and Certification of Xylazine-d6 for Equine Medication Regulation. Proceedings of the 21st International Conference of Racing Analysts and Veterinarians, Uruguay 2016, 21(1), 102–106.

Personal Contribution: Development of synthetic scheme based on previous work from same laboratory on the synthesis of deuterated hydroxyxylazine, sourcing of materials, benchwork/wet chemistry (under guidance of Dr. Sucheta Kudrimoti), assisted in proton-NMR work for confirmation of product, wrote manuscript.

SYNTHESIS, PURIFICATION AND CERTIFICATION OF XYLAZINE-d6 FOR EQUINE MEDICATION REGULATION

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ABSTRACT

Xylazine, N-(2,6-Dimethylphenyl)-5,6-dihydro-4H-1,3- thiazin-2-amine, is a short-acting alpha-2 agonist tranquilizer approved for therapeutic use in horses. Xylazine is also an Association of Racing Commissioners International [ARCI] Controlled Therapeutic Medication [CTM] with potential to affect equine performance. As such, in the United States its use in competition horses is regulated via a regulatory threshold in plasma for parent xylazine. We have therefore synthesized, purified and certified xylazine-d6, a deuterated analogue of xylazine, for use as an internal standard in quantification of xylazine. Xylazine-d6 was prepared commencing with commercially available 2,6-dimethyl-d6- aniline, which was reacted with thiophosgene to yield the isothiocyanate intermediate. Reaction of the isothiocyanate intermediate with 3-amino-1-propanol, followed by refluxing in concentrated HCl produced xylazine-d6 in moderate yield (40%) which was then purified and fully characterized by NMR, HPLC, GCMS and high-resolution mass spectroscopy for reference standard certification.

KEYWORDS

Xylazine-d6, internal standard, synthesis, purification, certification

INTRODUCTION

Xylazine, [N-(2,6-Dimethylphenyl)-5,6-dihydro-4H-1,3- thiazin-2-amine, C12H16N2S, Molar mass 220.334], is a rapidly acting alpha-2 agonist analgesic and tranquilizer that is widely used as a short acting tranquilizer in equine medicine (Greene & Thurmon 1988; Tobin et al., 2012). With this in mind, it has been deemed to have the

potential to affect performance in racehorses (Wagner et al., 1991). Previously xylazine has been regulated via the quantitation of its primary urinary metabolite, 4-hydroxyxylazine (Spyridaki et al., 2004; Tobin et al., 2012).

Within the United States, regulations restrict the presence of the parent compound (xylazine) in post-race plasma testing to no greater than 200 pg/mL in blood. Such decision limits are determined in various ways around the world. In many countries these are based upon 6 horse pharmacokinetic studies (IFHA, 2016) and the resulting decision limits are termed screening limits. In the United States, a similar strategy is invoked with the use of thresholds. In the context of this paper, threshold is intended to mean a concentration of a non-endogenous, therapeutic substance in an equine biological specimen below which the sample will not be considered to contain that substance in breach of the rules.

The recommended dose for xylazine is approximately 1 mg/kg, usually administered by rapid intravenous injection. Administered in this way, the plasma concentration of xylazine initially declines rapidly. This initial decline led to a regulatory threshold for xylazine to be set to 10 pg/mL in plasma in the United States but more recently this limit has been adjusted to 200 pg/mL in plasma, as this regulatory threshold value more appropriately reflects the drug's longer terminal half-life. Xylazine use has been approved by the US Food and Drug Administration in several species, most importantly in horses. Due to its ability to be medicinally used in racehorses, the Association of Racing Commissioners International [ARCI] has recognized it as a Controlled Therapeutic Medication [CTM] with potential to affect equine performance.

As earlier approaches to xylazine regulation involved use of the equine urinary metabolite 4-hydroxyxylazine [4-OH- xylazine] as the regulatory analyte, we have previously synthesized and certified both 4-OH-xylazine and its deuterated analogue (Kudrimoti et al. 2014). More recently, however, the preferred regulatory regime has favored the testing of plasma over urine and consequently the target analyte has shifted from the metabolite to parent drug. During the 20th ICRAV meeting in Mauritius, the authors were asked to consider the synthesis of a deuterated xylazine standard to complement the deuterated metabolite. To accommodate the request and change in methodology, the preparation and characterization of [2H6]-xylazine (xylazine-d6) is described herein.

SYNTHESIS

Synthesis of N-(2,6-Dimethyl-d6-phenyl)-5,6- dihydro-4H-1,3-thiazin-2-amine (Xylazine-d6):

Synthesis of 2,6-d6-dimethylaniline

Sulphanilic acid (1) (5.19 g, 30 mmol) and sodium carbonate (1.8 g, 17 mmol) were dissolved in 50 mL of hot (60^{o} C) water, cooled and sodium nitrite (2.1 g, 30 mmol) was slowly added portion-wise. The resultant mixture was left stirring for 1 hour and then poured onto 37 % (w/w) hydrochloric acid (6 mL) and ice (30 g), which was left to stir for another hour, yielding the required diazonium intermediate (2).

Next, commercially available 1,5-d6-dimethylxylene ((3), 3.6 g, 30 mmol) was dissolved in hot 2.5 M sodium hydroxide (20 mL) and the resultant basic solution poured into the acidic solution (2) containing the diazonium intermediate. This solution was left to stir for 1 hour at room temperature, yielding a red slurry which was heated to 55 °C and sodium dithionite (22.8 g, 130 mmol) was slowly added until the color turned yellow. The material was left stirring overnight at room temperature and the precipitated product was isolated via filtration to obtain 2,6-d6-dimethylaniline (4) as a yellow powder.

Preparation of 2,6-d6- dimethylphenylisothiocyanate (5):

The above 2,6-d6-dimethylaniline (4) (0.8 g, 4.47 mmol) was dissolved in dichloromethane (10 mL) followed by the addition of triethylamine (0.1 mL, 0.75 mmol) and thiophosgene (0.6 mL, 7.83 mmol) under ice-cooled conditions over 20 minutes. The resulting solution was allowed to stir for 1 hour at room temperature before being poured over ice (50 g) and extracted with dichloromethane to obtain 2,6-d6-dimethylphenylisothiocyanate (5) as a crude orange powder (yield 90%).

Preparation of xylazine-d6 (6):

2,6-d6-dimethylisothiocyanate (5) (1.70 g, 9.50 mmol) was dissolved in a solution of dichloromethane (20 mL) and 3-aminopropanol (1.70 mL, 1.67 g, 22.18 mmol). The solution was set to reflux overnight with stirring. The following morning it was cooled to room temperature and the solvent was evaporated using a rotary evaporator. Concentrated hydrochloric acid (8 mL) was added to the product and it was allowed to reflux and stir

overnight once again. The solution was then poured into an aqueous solution of 10 % sodium hydroxide (50 mL) and stirred for 3 hours. The final product (xylazine-d6, 6) precipitated as a hydrochloride salt, which presented as an off-white/yellow powder.

All organic extracts were dried with sodium sulphate, filtered, and concentrated on a rotary evaporator.

Figure 2.1.1.1 Xylazine-d6 Synthesis

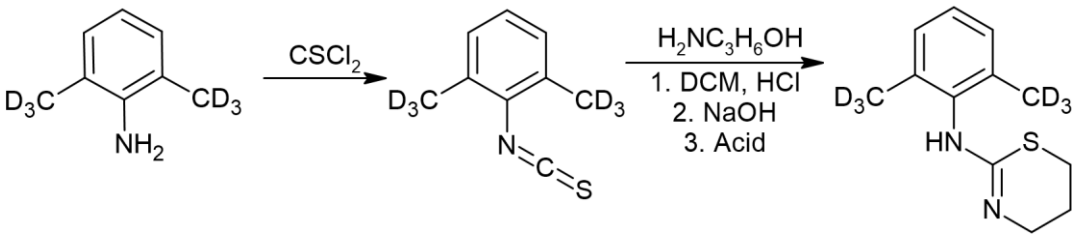

Figure 1: Xylazine-d6 Synthesis shows the reported synthetic pathway.

CHARACTERISATION

General

NMR spectra were measured on a Varian Unity Inova 400 MHz (CA, USA) spectrometer and chemical shifts are reported in ppm relative to DMSO-d6 as internal standard.

GCMS spectra were obtained on an Agilent 6890 GC- 5973 MSD (CA, USA) with a Zebron ZB-5MSi 15 m x 0.25 mm I.D. capillary column with a 0.25 µm film thickness (Phenomenex, Torrence, CA, USA). Helium carrier gas was used. The injector temperature was 250°C, with an injection concentration of 0.1 mg/mL and 1 µL injected with an autosampler. Electronic Pressure Control (EPC) was used at a constant flow rate of 1.0 mL/min carrier gas. Detection was with EI ionization operating in scan mode, 20-550 amu scan range with an acquisition rate of 4 samples per second. The detector temperatures were source at 250°C and quadrupole at 150°C.

HPLC UV detection was carried out with an Agilent 1100 HPLC (CA, USA) equipped with a column heater, diode array detector and an autosampler. A binary solvent system consisting of water with 0.1% formic acid and HPLC grade acetonitrile (Pharmaco-Aaper, KY, USA) with 0.1 % formic acid was used with a Phenomenex Kinetex C18 HPLC

column (150 mm x 2.1 mm, 2.6 µm particle with 100 Å pore size) protected with a matched Phenomenex C18 guard cartridge at a constant column temperature at 30°C.

Xylazine was analyzed by injection of 5 µL of a solution at 0.1 mg/mL concentration. The initial solvent mixture used a linear gradient of 5% to 95% acetonitrile gradient over 12 minutes after a 2 min isocratic holding time at the initial conditions and re-equilibration at the initial conditions for 10 minutes. The flow rate was 0.18 mL/min. The diode array detector was monitored at 210 nm.

Combustion analyses were carried out by Atlanta Microlabs, Atlanta, GA.

Mass spectra were acquired on a Thermo Scientific Q Exactive Orbitrap mass spectrometer (Bremen, Germany). Typically, samples were dissolved in 50% acetonitrile: 50% water and infused by syringe pump into the electrospray source at 5 uL/min. Spectra were acquired at 140,000 mass resolution in the positive ion mode.

Crystallization of the crude product produced an off-white powder from ether-hexane with melting point 137-138ºC; 1H-NMR (400MHz, DMSO-d6): δ 2.01 (m, 2H), 2.86 (t, 2H), 3.31 (t, 2H), 6.90 (t, 1H), 7.0-7.01 (d, 2H). Characterization data are further summarized in Table 1.

High resolution ESI-MS confirmed the expected M+1 molecular formula: C12H 2H6N2S, theoretical m/z 227.1484, observed m/z 227.1483, delta mass -0.4486 ppm. The corresponding unlabeled xylazine was also analyzed by High Resolution ESI-MS as a comparison to the labelled material which corresponded to the expected M+1 molecular formula: C12H17N2S, theoretical m/z 221.1107, observed m/z 221.1106, delta mass -0.6214 ppm.

Combustion analysis revealed the carbon, hydrogen (corrected for deuterium mass), sulphur, and nitrogen percentages to be consistent with the calculated molecular formula.

Expected: Percent (%) C, 63.67; H+D, 7.12; N, 12.38; S, 14.16

Found: C, 63.70; H+D, 6.94; N, 12.37; S, 14.38

EI-GCMS indicated a single peak and an apparent molecular ion at m/z 226, consistent with the expected molecular mass. Isotopic distribution analysis showed the following deuterated compositions: d0, d1, d2, d3 – 0%, d4 – 1.61%, d5 – 5.84%, d6 – 92.54%. 1H-NMR taken in DMSO-d6 indicated

that the structure was consistent with xylazine-d6.

Table 2.1.1.1 Characterization Data of Xylazine-d6

Appearance	Off-white powder	
Chemical Formula	$C_{12}H_{10}D_6N_2S$	
Exact Mass	227.1484 (Calculated)	227.1483 (Found) Δ mass 0.6214 ppm
Combustion Analysis.	C, 63.67; H, 7.12; N, 12.38; S, 14.16 (Calculated)	C, 63.70; H, 6.94; N, 12.37; S, 14.38 (Found)
Volatiles (Headspace GC)	<0.1% volatiles	
Micro Ash	<0.1% residue on ignition	
Melting Point	137-138ºC	
Isotopic Purity	92.54% d_6, 5.84% d_5, 1.61% d_4, 0% d_0	
^{1}H-NMR (400MHz, DMSO-d_6) δ		

Table 1: Characterization data shows the tabulated values for the characterization acquired for xylazine-d6.

Figure 2.1.1.2 1H-NMR Spectra for Xylazine/Xylazine-d6

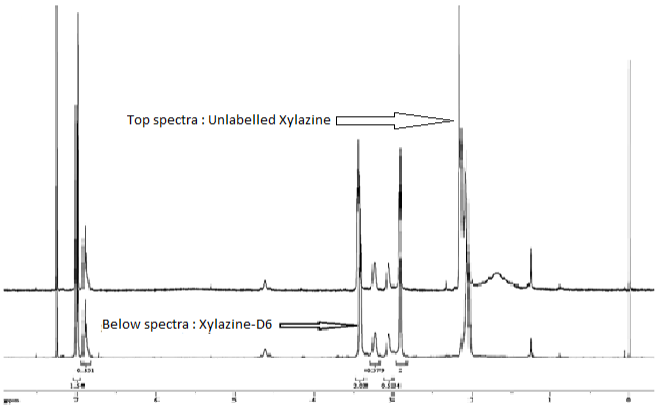

Figure 2: 1H-NMR Spectra of unlabeled and labelled xylazine shows the overlaid spectra, with the deuterium free spectrum located above the d6-labeled spectrum. The conspicuous absence of the 2,6-dimethyl signal at 2.1ppm indicates incorporation of deuterium at the target site.

CONCLUSION

This synthesis is based on previous work from our group that described the synthesis of the labelled phase 1 metabolite of xylazine, namely 4-hydroxy-xylazine. The primary difference in this procedure was use of 2,6-dimethyl-d6-aniline as the starting material rather than 4-hydroxy-2,6-d6-dimethylaniline, as was used in the previous 4-hydroxyxylazine synthesis.

Following the procedure described above, xylazine-d6 was synthesized commencing with 2,6-d6-dimethylaniline in dichloromethane via reaction with thiophosgene in the presence of triethylamine, yielding 2,6-d6-dimethylphenylisothiocyanate. This intermediate was then reacted with 3-amino-1-propanol in dichloromethane. After work-up and extraction, the final product was crystallized as an off-white powder from ether-hexane.

Successful synthesis of the deuterated compound was then verified via a comparison of 1H-NMR to the non- deuterated compound. The noteworthy 2,6-dimethyl resonance that appears at 2.1 ppm is absent in the deuterated compound, while all other peaks remain present.

Herein reported is a purified and characterized deuterated xylazine suitable for use as an internal standard. The characterization of xylazine-d6 meets industry requirements for chemical identity, spectroscopic and chemical purity in compliance with ISO analytical standards.

AUTHOR'S DECLARATION OF INTERESTS

Jake Machin and Kimberly Brewer are students who contributed to and participated in the research work. Sucheta Kudrimoti, Rodney Eisenberg and Thomas Tobin are doctoral level researchers who contributed to the conception, implementation, performance, and certification of the deuterated xylazine. The basic chemical synthesis work was performed in the Department of Veterinary Science of the Maxwell H. Gluck Equine Research Center at The University of Kentucky with intellectual and or hands-on input from all authors. The synthesized compound was then transferred under a University of Kentucky Intellectual Property (IP) Technology Transfer License to Frontier BioPharm, LLC, PO Box 614, Richmond, Kentucky, 40476 USA where it was chemically purified, characterized, forensically certified and prepared for worldwide distribution as a Certified Reference Standard. This licensing and technology transfer arrangement is necessary since the University of Kentucky, as a non- profit academic institution, does not certify and market entities such as these certified reference standards. The University of Kentucky owns the Intellectual Property associated with the identification and synthesis of these reference standards and Drs. Eisenberg and Tobin are listed as creators of the relevant University of Kentucky IP. As such, Drs. Tobin and Eisenberg may benefit from an inventor's portion of any royalty income to UK under the relevant IP/Technology Transfer license and Dr. Eisenberg has a founder's ownership interest in Frontier BioPharm, LLC.

ACKNOWLEDGEMENTS

Published as paper 483 from the Equine Pharmacology, Therapeutics and Toxicology Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky. The information reported in this paper is part of a project of the Kentucky Agricultural Experiment Station (KAES manuscript # 17- 14-077) and is published with the approval of the Director. This work was made possible by research support from The National Horsemen's Benevolent and Protective Association and the Alabama, Arizona, Arkansas, Canada, Charles Town (West Virginia), Florida, Iowa, Indiana, Kentucky, Louisiana, Michigan, Minnesota, Nebraska, Ohio, Oklahoma, Ontario (Canada), Oregon, Pennsylvania, Tampa Bay Downs (Florida), Texas, Washington State, and West Virginia Horsemen's Benevolent and Protective Associations and the Florida Horsemen's Charitable Foundation, the Oklahoma Quarter Horse Racing Association, Neogen Corporation and the Equine Health and Welfare Alliance, Lexington, Ky.

REFERENCES

Choo H.Y. P. & Choi S. O. (1991) The metabolism of xylazine in rats Anal Pharm Res 14(4), 346-251

Garcia V. R., Toutain P. L., Alveinerie M. & Ruckebusch

Y. (1981) The pharmacokinetics of xylazine hydrochloride: an interspecific study Journal of Veterinary Pharmacology and Therapeutics 4, 87–92

Greene S. A. & Thurmon J. C. (1988) Xylazine – a review of its pharmacology and use in veterinary medicine Journal of Veterinary Pharmacology and Therapeutics 11, 295–313

Kudrimoti S., Eisenberg R., Maylin G. A., Hughes C. & Tobin.T. (2014) Synthesis and characterization of 4-hydroxyxylazine and 4-hydroxyxylazine-d6 standards for use in xylazine regulation in competition horses Proc 20th Int Conf Racing Anal Vet, 332-335

Spyridakia M. H., Lyrisa E., Georgoulakisb I., Kouretasb D., Konstantinidouc M. & Georgakopoulosa C.G. (2004) Determination of xylazine and its metabolites by GC–MS in equine urine for doping analysis J Pharm Biomed Analysis 35(1), 107-116.

Tobin T., Brewer K. & Stirling K. H. (2012) World Rules for Equine Drug Testing and Therapeutic Medication Regulation. Nicholasville, Kentucky. Wind Publications.

Tobin T. & Charles C. T. (1981) Drugs and the Performance Horse, Charles C Thomas Pub Ltd
ISBN-10: 0398044465
Wagner A. E., Muir W. W. & Hinchcliff K. W. (1991) Cardiovascular effects of xylazine and detomidine in horses. Am J Vet Res 52(5), 651-657

2.1.2 Synthesis and Characterization of Barbarin, A possible Source of Unexplained Aminorex Identifications in Forensic Science

Abstract: Aminorex is a US DEA Schedule 1 controlled substance occasionally detected in racing horses. A number of aminorex identifications in sport horses were thought to have been caused by exposure to plant sources of aminorex. Glucobarbarin, found in plants of the Brassicaceae family, has been suggested as a potential proximate chemical source by being metabolized in the plant or the horse to aminorex. In Brassicaceae, glucobarbarin is hydrolyzed by myrosinase to yield barbarin, which serves as an insect repellant and/or attractant and is structurally related to aminorex. We now report the synthesis, purification and characterization of barbarin for use as a reference standard in aminorex related research concerning equine urinary identifications of aminorex and also for possible use in equine administration experiments. Synthesis of barbarin was performed via ring closure between phenylethanolamine and carbon disulfide in tetrahydrofuran with the catalyst pyridine at reflux. The reaction yielded a white crystalline substance that was purified and chemically characterized as barbarin for use as a Certified Reference Standard or for studies related to equine aminorex identifications.

Introduction: Aminorex is a DEA Schedule I substance with amphetamine-like properties. Initially available as an anorectic in Europe, it was withdrawn from the market when it was linked to deaths related to pulmonary hypertension. The Association of Racing Commissioners International specify aminorex as a Class 1, Penalty class A foreign substance, indicating major penalties for those violating its prohibition. However, it has in the past been shown to present in urine when horses are treated with the anthelmintic drug levamisole. Presence of its cometabolite rexamino in the urine can be used to verify this defense, but sporadic positives at low levels continue to be identified even when levamisole is not an explanatory agent, indicating a likely environmental source of the substance.

The plant substance barbarin was suggested by Voss in 2018 as a potential source of aminorex, and a study undertaken by Maylin, et al showed that the parent plant (*B.*

vulgaris) induced aminorex in urine when fed to thoroughbreds. Traditional synthesis of barbarin is neither economical nor efficient, as it relies on the lysing of cells from the plant and allowing the hydrolyzation of glucobarbarin by myrosinase. With that in mind, the need for a Certified Reference Standard of synthetic barbarin was deemed necessary and a synthetic pathway developed.

Experimental: Barbarin was synthesized via the ring closure of 2-hydroxy-2-phenylethylamine in tetrahydrofuran with catalytic pyridine and excess carbon disulfide. The mixture was allowed to reflux overnight, and the completion of the reaction was tracked via Thin-Layer Chromatography in a 19:1 dichloromethane: methanol solution. After the reaction was completed, the mixture was concentrated, diluted with dichloromethane, and washed with dilute hydrochloric acid, brine, and dried over sodium sulfate. Following filtration, the solution was concentrated under vacuum and then purified by re-crystallization to yield the resultant white crystalline material.

The resultant material was characterized via Proton Nuclear Magnetic Resonance, High Resolution Mass Spectrometry, and Combustion Analysis, indicating barbarin at a purity of 99.7% purity.

Conclusion: Barbarin is now available as a Certified Reference Standard via a scalable synthetic pathway for the first time.

Citation: Machin, J., Childers, T., Kudrimoti, S., Eisenberg, R., Fenger, C., Hartmann, P., ... & Tobin, T. (2020). Synthesis and characterization of barbarin, a possible source of unexplained aminorex identifications in forensic science. Drug Testing and Analysis, 12(10), 1477-1482.

Personal Contribution: Sourcing of materials, benchwork/wet chemistry, assisted in proton-NMR work for confirmation of product, developed proposed mechanism of ring closure, wrote manuscript.

SYNTHESIS AND CHARACTERIZATION OF BARBARIN, A POSSIBLE SOURCE OF UNEXPLAINED AMINOREX IDENTIFICATIONS IN FORENSIC SCIENCE

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ABSTRACT:

Aminorex is a US DEA Schedule 1 controlled substance occasionally detected in racing horses. A number of aminorex identifications in sport horses were thought to have been caused by exposure to plant sources of aminorex. Glucobarbarin, found in plants of the Brassicaceae family, has been suggested as a potential proximate chemical source by being metabolized in the plant or the horse to aminorex. In Brassicaceae, glucobarbarin is hydrolyzed by myrosinase to yield barbarin, which serves as an insect repellant and/or attractant and is structurally related to aminorex. We now report the synthesis, purification and characterization of barbarin for use as a reference standard in aminorex related research concerning equine urinary identifications of aminorex and also for possible use in equine administration experiments. Synthesis of barbarin was performed via ring closure between phenylethanolamine and carbon disulfide in tetrahydrofuran with the catalyst pyridine at reflux. The reaction yielded a white crystalline substance that was purified and chemically characterized as barbarin for use as a Certified Reference Standard or for studies related to equine aminorex identifications.

KEYWORDS: Aminorex, Barbarin, Synthesis, Brassicaceae, Equine Forensic Chemistry

INTRODUCTION:

Aminorex, (RS)-5-Phenyl-4,5-dihydro-1,3-oxazol-2-amine, molar mass, 162.19, (Figure 1), is an amphetamine-like substance that was at one time marketed in Europe as an anorectic for weight control. Aminorex, however, was soon associated with deaths from pulmonary hypertension (Poos et al, 1963) and withdrawn from marketing in 1972. Aminorex is a more potent anorectic than d-amphetamine and its 4-Methylaminorex analogue has central effects comparable with those of methamphetamine and both are available as illicit recreational substances. Aminorex is a US DEA Schedule 1 controlled substance and an Association of Racing Commissioners International [ARCI] Class 1, Penalty class A foreign substance, so findings of aminorex in post event equine samples can give rise to significant penalties for horsemen (ARCI Uniform Classification Guidelines for Foreign Substances January 2018 (V.13.4)).

Figure 2.1.2.1 Structure of Aminorex

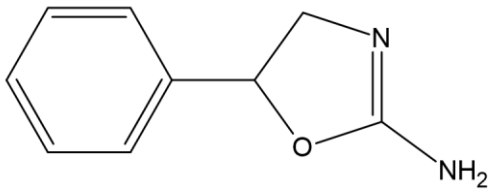

Figure 1: Aminorex, (RS)-5-Phenyl-4,5-dihydro-1,3-oxazol-2-amine, molar mass, 162.19,

A sequence of aminorex identifications in US racing, starting in 2002 (Sams 2006, Soma et al 2008, Barker 2009), was shown in 2007 to be the result of administration of Levamisole, an anthelmintic and immune stimulant which horses and humans metabolize to aminorex (Loganathan et al 2009, Ho et al 2009. Gutierrez et al 2010, Hofmaier et al 2014). Identification of this unexpected source of aminorex reduced the frequency of aminorex identifications in horse-racing but did not eliminate them. Unexplained aminorex identifications continued, sometimes appearing as “clusters” of identifications, including most recently in a number of sport horses with no history or analytical evidence of exposure to either Levamisole, Tetramisole or aminorex itself (Teale and Biddle, 2018).

Possible Botanical Sources for Aminorex

Reviewing the analytical data from these sport horse urine samples a number of small nitrogenous molecules were identified, along with aminorex. Based on this analysis, the plant substance barbarin as has been suggested (Voss, 2018) as a potential source of aminorex. Plants of the genus Barbarea, Brassicaceae family contain glucobarbarin, a barbarin precursor. In these plants glucobarbarin is hydrolyzed by myrosinase to an intermediate which spontaneously cyclizes to yield barbarin, Figure 2, which functions as an insect repellant or attractant when the plant structure is damaged (Kjaer et. al., 1957). Barbarin is structurally related to aminorex, so it is possible that either glucobarbarin or barbarin or a chemically related substance in Brassicaceae fragments in equine feedstuffs may be a source of these unexplained aminorex identifications in horse urine.

Because of the structural similarities between barbarin and aminorex and the possible role of glucobarbarin or barbarin as an aminorex precursor in herbivore diets, we have synthesized, purified, and certified barbarin with the goal of making it available as a Certified Reference Standard and also as a possible equine administration substance. Availability of chemically pure barbarin will allow investigation of the role of barbarin as a possible botanical source of aminorex in the matter of unexplained post-race aminorex identifications in equine forensic samples, and in such cases where it may serve as a biomarker of botanical sources of aminorex (Machin et al 2018).

Figure 2.1.2.2 Natural Conversion of Glucobarbarin to Barbarin

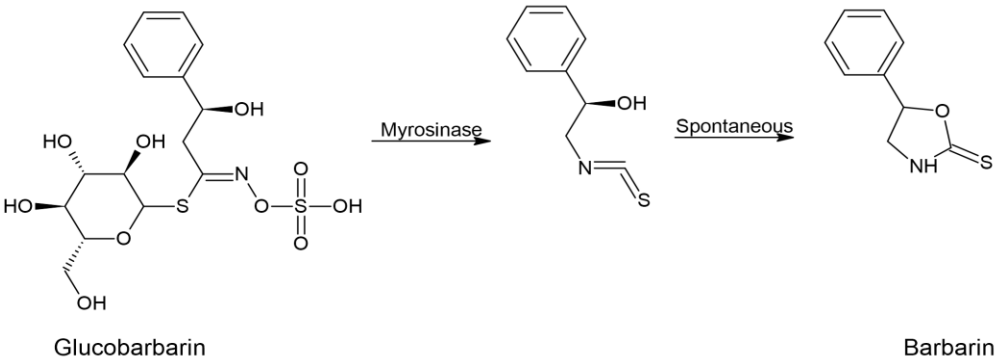

Figure 2: Glucobarbarin, above left, hydrolyzed by the enzyme myrosinase to the intermediate, above center, which then spontaneously cyclizes to barbarin, 5-phenyl-2-oxazolidinethione, C9H9NOS,179.24g/mol, above right, structurally related to aminorex, as shown.

EXPERIMENTAL:

1/ Barbarin Synthesis

Barbarin, ((RS)-5-Phenyl-2-oxazolidinethione: C9H9NOS, molar mass:179.237 g/mol) was prepared based on the previous synthesis of related substances (Santoro et al,1976) and personal communications with Dr. Richard Sams. Synthesis commenced by taking a solution of 2-hydroxy-2-phenylethylamine (1.0 g, 1 eq.) in tetrahydrofuran [THF] (20 mL) and to which was added pyridine (0.59 ml, 1 eq.) and excess carbon-disulfide (6 ml). The mixture was then refluxed overnight at 70°C. When the synthesis reaction was complete as determined by Thin-Layer Chromatography (19:1 dichloromethane (DCM): methanol) the reaction mixture was concentrated, diluted with DCM, and washed with dilute HCl, brine, and dried over sodium sulfate. Following filtration, the solution was concentrated under reduced pressure and then purified by crystallization (9:1 DCM: n-hexanes) to yield the resultant white crystalline material.

Figure 2.1.2.3 Proposed Mechanism for Ring Closure of Barbarin Synthesis

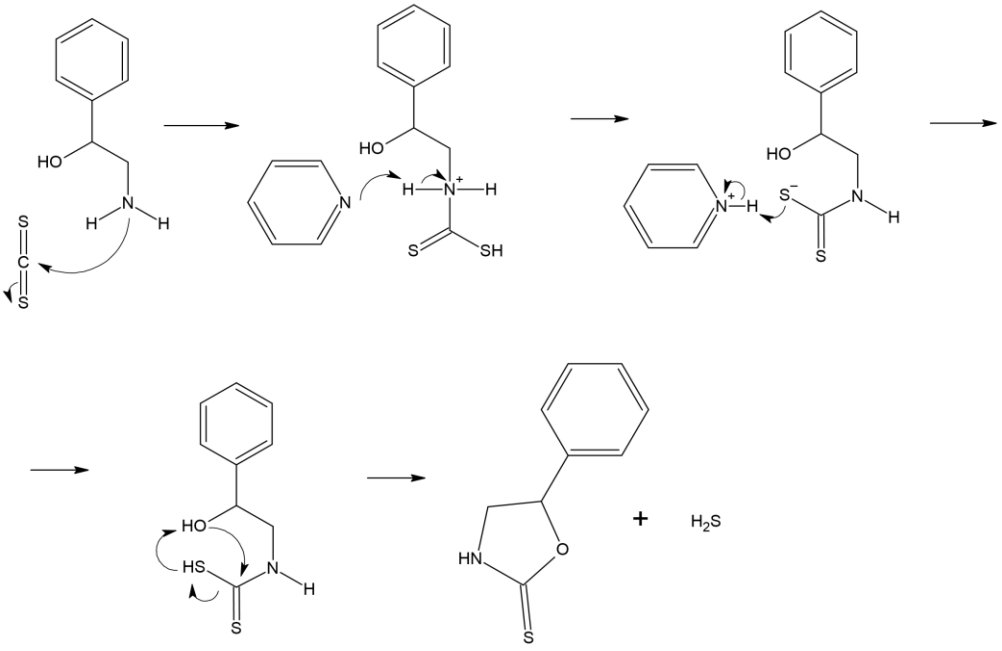

Figure 3: Proposed mechanism for synthesis of barbarin from phenylethanolamine andcarbon disulfide with pyridine catalyst. Nucleophilic attack of the amino group on carbon disulfide results in ring closure, with pyridine acting as a proton shuttle.

2/ *Barbarin characterization:*

The white crystalline material resulting from the above-described reactions was chemically characterized as barbarin by 1/ Proton nuclear magnetic resonance, 2/ High Resolution Mass Spectrometry, and 3/ combustion analysis, as follows:

Figure 2.1.2.4 1H-NMR Analysis for Barbarin Product

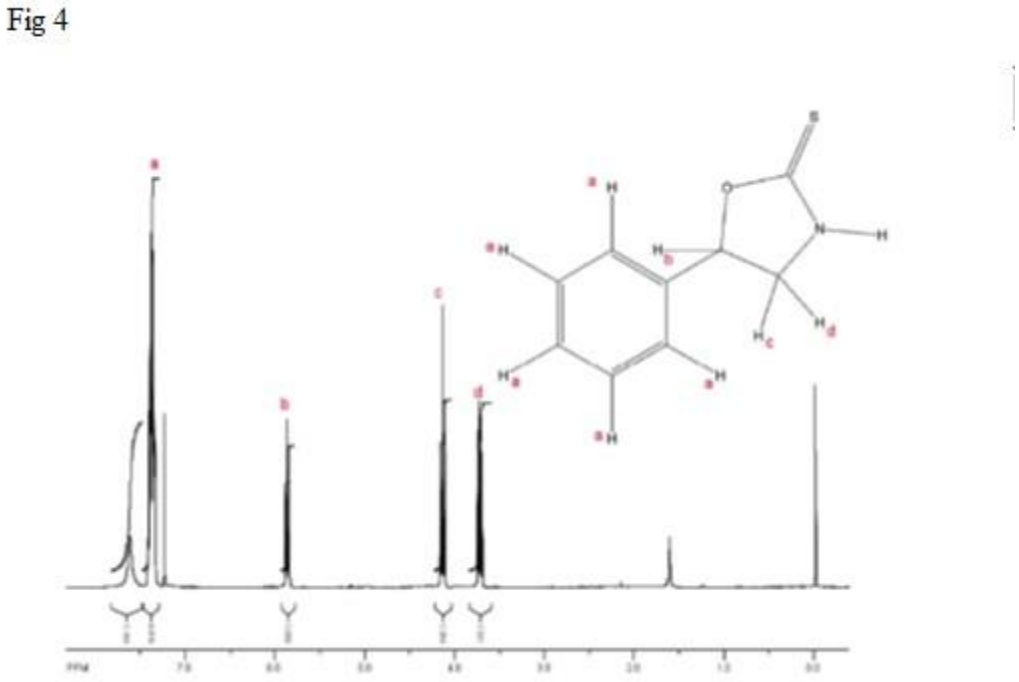

Figure 4: 1H-NMR analysis 1/ 1H-NMR, (CDCl3,400 MHz) δ (ppm): 7.62 (br s, 1H), 7.35-7.45 (m, 5H), 5.87 (t, 1H), 4.15(dd, 1H). 3.74(dd, 1H)

Aromatic protons showing up at 7.35-7.45 ppm as multiplet and the proton Hb of five membered ring as a triplet at 5.87 ppm, the Hc proton as double doublet at 4.15pm and Hd proton as double doublet at 3.74 ppm and protonated molecule ion peak m/z 180.0477 in high resolution mass spectrum confirms the structure of barbarin.

Figure 2.1.2.5 High-Resolution Mass Spectral Analysis of Barbarin Product

Fig 5

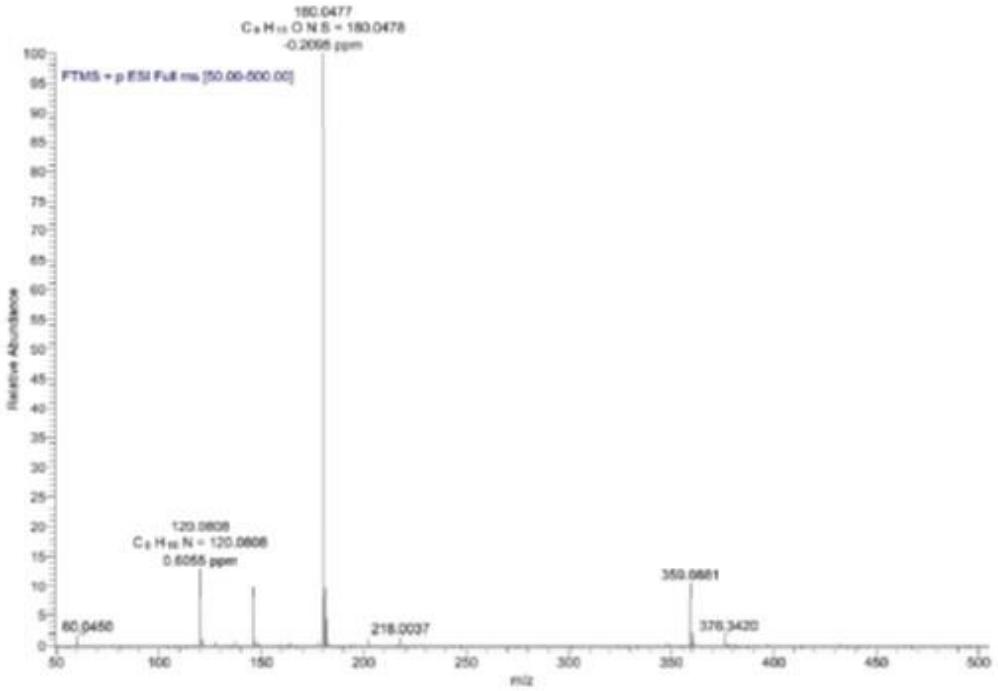

Figure 5: High-Resolution Mass Spectral analysis (ESI, M+1) found: C9H10NOS 180.0478.

Peaks are believed to represent as follows: m/z 180, barbarin + H+; m/z 359, barbarin noncovalent dimer + H+; m/z 120, barbarin oxazolidinethione ring cleavage product; m/z 146 = barbarin loss of SH group (S plus a neighboring H). Reversed phase HPLC UV detection (C18, methanol water gradient diode array UV detection) indicated a purity of approximately 99.5%.

Another area of regulatory significance is the chirality of barbarin and or aminorex originating via the proposed glucobarbarin / barbarin pathway. Review of the literature shows that plant barbarin is predominantly found as the R isomer, suggesting that aminorex originating from plant barbarin is also likely the be found as the R isomer. Given that synthesized aminorex is likely to be racemic, a urinary identification of a small concentration of predominantly R aminorex would be entirely consistent with the aminorex

identification being of botanical origins, as previously demonstrated to be valuable in regulatory evaluation (Barker, 2009).

Combustion analysis CHNS, Calc: C, 60.31%, H, 5.06%, N, 7.81%, S, 17.89%. Found: C 60.16%, H 4.97%, N 7.77%, S 17.89%, consistent with 99.7% purity.

DISCUSSION:

The synthesis described in methods proceeded as described and produced a 75% yield of a white crystalline substance. This reaction product was purified by recrystallization to a chemical purity of 99.5% and the purified product was shown to be barbarin suitable for use as a Certified Reference Standard or for equine administration experiments. Further work was designed to determine whether or not barbarin was/is the actual proximate source of aminorex such as we identified when the Brassicaceae plant Barbarea vulgaris, colloquially “Yellow Rocket”, was consumed by horses and aminorex identified in the post administration urine samples, as described below.

In spring 2018, responding to regulatory concerns (Angst, April 17, 2018; Voss, April 17, 2018), we harvested flowering Kentucky Barbarea vulgaris and sent it to colleagues in New York for equine administration. The experimental horses declined consumption when offered the dried plant materials, but readily consumed the plant material when mixed with sweet feed. Then, four hours post consumption of Barbarea vulgaris showed the presence of aminorex in their urine samples with no evidence for the presence of barbarin in the same urine (Maylin, et al 2019).

At this time, however, it is unclear as to what precisely is the proximate chemical source of these post Brassica vulgaris administration urinary aminorex identifications. Whatever the mechanism, however, reviewing a number of apparently low concentrations aminorex identifications in post-race samples, the Kentucky Horse Racing Commission chose to rescind a “positive” identification at their April 17, 2018, meeting in Lexington, based on uncertainties concerning the origins of the aminorex identifications (Angst, 2018; Voss, 2018). In particular, it appears that as well as aminorex, barbarin was identified in

the samples from the horse, giving rise to the possibility that the aminorex identification was due to inadvertent exposure of the horse to a plant source of barbarin.

Our initial working hypothesis was that barbarin formed in the Brassicaceae plant associated with harvesting damage to the plant was the likely proximate source of aminorex, as set forth in Figure 2. In this model barbarin is present in the plant on ingestion and is metabolically transformed in/by the horse to aminorex which is then excreted in the urine, leading to urinary aminorex identifications. In this model the metabolic transformation from barbarin to aminorex occurs in the horse and is driven by the metabolic capabilities of the horse (Tobin, 1981). An alternative hypothesis is that the barbarin is excreted in the urine and that the transformation from barbarin to aminorex occurs in the urine sample (Voss, 2018), although this seems a less likely possibility.

The fact that consumption of Brassicaceae plants is associated with aminorex identifications in equine urine has significant scientific and forensic implications. Aminorex, a substance closely related to Amphetamine has been identified in equine urine samples without evidence of exposure to Levamisole. Between the years of 2016 and 2018, 11 unexplained low level aminorex results have been detected in US sport horses: 3 in Illinois between 2016 and 2017, 5 in Massachusetts in 2017, 3 more in Kentucky in 2018, and 1 more in 2019 (Holloway, 2019). A proposed source of such urinary aminorex identifications is an equine driven metabolic transformation of barbarin or a related substance from plants of the Brassicaceae family inadvertently entering equine feedstuffs (Teale and Biddle, 2018) to aminorex, as has previously been shown to occur following Levamisole administration. Experimental approaches to this question were hindered by the lack of availability of chemically pure barbarin, for use both as a research reference standard and also in amounts sufficient for equine administration and related experiments. Other plants of potential interest include Reseda luteola (colloquially Dyer's Rocket), a known producer of glucobarbarin, and the common Cardamine hirsute (colloquially hairy bittercress).

Barbarin made possible by this research project will allow more in-depth investigation of the potential role of glucobarbarin and related substances as chemical precursors of aminorex in horses., as recently demonstrated by Machin et al, 2018, and possibly also in other mammalian metabolic systems. As such availability of analytical

standard quality Barbarin in amounts sufficient for animal administration will be useful in the field of equine forensic science.

CONCLUSIONS:

We now report synthesis, purification, and characterization of the plant glucosinolate barbarin in gram quantities. Barbarin, a white crystalline substance is now available as a certified reference standard for analytical/forensic toxicological research. Additionally, this synthetic method described is readily capable of producing larger quantities of Barbarin, such as may be required for equine administration or similar in vivo experiments (Machin et al 2018, Tobin, 1981).

ACKNOWLEDGEMENTS:

This research was made possible by research support from The Equine Health and Welfare Alliance, Inc, Versailles, Kentucky, and the United States Trotting Association, Columbus, OH. Further support came from the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch Program under project KY014051 Accession Number 1010609 and also by grants from the USDA Agriculture Research Service Specific Cooperative Agreement #58-6401-2-0025 for Forage-Animal Production Research, the Kentucky Department of Agriculture and the Kentucky Thoroughbred Association Foundation and by support for the Kentucky Agricultural Experiment Station as provided by the National Institute of Food and Agriculture (NIFA) and the Commonwealth of Kentucky. Other support includes research support from The National Horsemen's Benevolent and Protective Association and the Alabama, Arizona, Arkansas, Ontario, Canada; Charles Town, WV; Florida, Indiana, Iowa, Kentucky, Louisiana, Michigan, Minnesota, Nebraska, Ohio, Oklahoma, Oregon, Pennsylvania, Tampa Bay Downs, Florida, Texas, Washington State, and West Virginia Horsemen's Benevolent and Protective Associations. Published as paper #499 from T Tobin and the Equine Pharmacology, Therapeutics and Toxicology Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky.

LITERATURE CITED:

Angst, F. (2018) https://www.bloodhorse.com/horse-racing/articles/227038/with-too-many- unknowns-kentucky-rescinds-drug-positive April 17, 2018. Accessed May 21, 2020 ARCI Uniform Classification Guidelines for Foreign Substances January 2018 (V.13.4).

Barker, S. A. (2009). The formation of Aminorex in racehorses following levamisole administration. A quantitative and chiral analysis following synthetic Aminorex or levamisole administration vs. Aminorex-positive samples from the field: a preliminary report. Journal of veterinary pharmacology and therapeutics, 32(2), 160-166.

Brewer, K., Shults, T. F., Machin, J., Kudrimoti, S., Eisenberg, R. L., Hartmann, P, and Tobin, T. (2016). A cluster of trace-concentration methamphetamine identifications in racehorses associated with a methamphetamine-contaminated horse trailer: A report and analysis. The Canadian Veterinary Journal, 57(8), 860.

Eiden, C., Peyrière, H., Diot, C., & Mathieu, O. (2015). Prevalence of levamisole and Aminorex in patients tested positive for cocaine in a French University Hospital. Clinical toxicology, 53(7),604-608

Gutierrez, J., Eisenberg, R. L., Koval, N. J., Armstrong, E. R., Tharappel, J., Hughes, C. G., &Tobin, T. (2010). Pemoline and Tetramisole 'positives' in English racehorses following levamisole administration. Irish veterinary journal, 63(8), 498.

Ho, E. N. M., Leung, D. K. K., Leung, G. N. W., Wan, T. S. M., Wong, A. S. Y., Wong, C.

H.F., ... & Sams, R. (2009). Aminorex and rexamino as metabolites of levamisole in the horse. Analytica chimica acta, 638(1), 58-68.

Hofmaier, T., Luf, A., Seddik, A., Stockner, T., Holy, M., Freissmuth, M., ... & Kudlacek, O. (2014). Aminorex, a metabolite of the cocaine adulterant levamisole, exerts amphetamine like actions at monoamine transporters. Neurochemistry international, 73, 32-41.

Holloway, K. (2019). Association of Racing Commissioners International Personal Communication

Kjaer, A., & Gmelin, R. (1957). IsoThiocyanates. XXVII. A New isoThyiocyanate Glucoside (GlucoBarbarin) Furnishing (-)-5-Phenyl-2-oxazolidinethione upon Enzymic Hydrolysis. L. Acta Chemica Scandinavica, 11, 906-907. doi: 10.3891/acta.chem.scand.11- 0906 Kjar, A., & Gmelin, R. (1958). IsoThiocyanates.

XXXIII. An isoThyiocyanate Glucoside (GlucoBarbarin) of Reseda luteola L. Acta Chemica Scandinavica, 12, 1693-1694. Doi: 10.3891/acta.chem.scand.12-1693

Loganathan, D., Fu, D., Zhao, S., Kwan, R., Chen, E., & Morrissey, B. (2009). Occurrence of Aminorex and Levamisole in Canadian Racing Part 2: Comparison of Levamisole and Aminorex Metabolism and Elimination Profiles in the Horse. In Proceedings of the 17th International Conference of Racing Analysis and Veterinarians. Antalya, Turkey.

Machin, J. Kudrimoti, S. Eisenberg, R., Fenger, C., Hartmann, P., Maylin, G., Tobin, T., and Shults, T. (2018). "Synthesis, Characterization and Certification of Barbarin, a Possible Botanical Source of Aminorex Identifications". Submitted for presentation and publication, the Society of Forensic Toxicology (SOFT) Annual Meeting, Oct 07-12, 2018. Minneapolis, Minnesota.

Maylin, G., Fenger, C., Machin, J., Kudrimoti, S., Eisenberg, R., Green, J., & Tobin, T. (2019). Aminorex identified in horse urine following consumption of Barbarea vulgaris, a preliminary report. Irish Veterinary Journal, 72(1), 15.

Poos, GI, Carson, JR., Rosenau, JD, et al. 2-Amino-5-aryl-2-oxazolines. Potent new anorectic agents. J Med Chem 1963; 6; 266-272.

Sams, R.A. (2006) Detection and Identification of Aminorex and Rexamino from Blood and Urine Samples from Racehorses. In Proceedings of the 16th International Conference of Racing Analysts and Veterinarians. Tokyo, Japan.

Santoro, R., Warren, R., & Roberts, G. (1976). Spontaneous formation of 4-methyl-5-phenyloxazolidine-2-thione from phenylpropanolamine. Journal of Chromatography A, 117(2),375-382.

Soma, L. R., Rudy, J. A., Uboh, C. E., Xu, F., & Snapp, H. M. (2008). Pharmacokinetics and effects of Aminorex in horses. American Journal of Veterinary Research, 69(5), 675-681.

Teale, P. and Biddle, S. (2018). Possible Plant Sources of Aminorex and Potential markers of Their Ingestion in the Horse. Proceeds of the 22nd International Conference of Racing Analysists and Veterinarians. Dubai, UAE in press.

Tobin, T. (1981). Drugs and the performance horse. Charles C. Thomas, Springfield Illinois,1981.

Voss N, (2018) https://www.paulickreport.com/news/the-biz/the-trouble- with-aminorex/ April 17, 2018. Accessed March 21, 2020

2.1.3 Synthesis and Characterization of d5-Barbarin for use in Barbarin Related Research

Abstract: Based on structural similarities and equine administration experiments, Barbarin, 5-Phenyl-2-oxazolidinethione from Brassicaceae plants is a possible source of equine urinary identifications of aminorex, (RS)-5-Phenyl-4,5-dihydro-1,3-oxazol-2-amine, an amphetamine related US Drug Enforcement Administration (DEA) substance

considered illegal in sport horses. We now report synthesis and certification of d5-

barbarin to facilitate research on the relationship between plant barbarin and such

aminorex identifications. D5-barbarin synthesis commenced with production of d5-oxime from d5-Acetophenone via butylnitrite in an ethoxide/ethanol solution. This oxime was then reduced with Lithium Aluminum Hydride to produce the corresponding alcohol-amine. Final ring closure of the alcohol amine was performed by addition of carbon disulfide with pyridine catalyst. The reaction product was purified by recrystallization and presents a stable white crystalline powder. This material was shown to be d5-barbarin by proton NMR, Mass Spectrometry, and Elemental Analysis and was purified and chemically characterized as 97% pure d5-barbarin for use as an Internal Standard in barbarin related equine forensic research.

Introduction: Based on structural similarities and equine administration experiments, barbarin is a primary candidate to induce the production of the DEA Schedule I substance aminorex in horse urine. Recent studies indicate that the consumption of *Barbarea vulgaris* can induce aminorex in horse urine but does not unequivocally determine barbarin as the chemical predecessor. The need for a deuterated standard fulfills two needs then: 1/ the quantification of barbarin in biological samples via use as an internal standard, and 2/ effective use of the labelled compound as a precursor in animal administration experiments to determine a direct linkage.

Experimental: The synthesis took place in three steps. The first step saw butyl nitrite dissolved in ice-cold ethanol, followed by the addition of sodium ethoxide. D5-

acetophenone was added to this solution (dissolved in ethanol) dropwise over 30 minutes. The solution was maintained at ice cold temperature until the addition was complete, at which time it was stirred until reaching room temperature and continued overnight. The following day the precipitate was filtered and washed with ether, dissolved in a minimal amount of water, acidified with glacial acetic acid, and the resultant white solid filtered and recrystallized with ethanol.

The second synthetic step was the reduction of the resulting d5-oxime via lithium aluminum hydride. This was done in anhydrous ether under argon at zero degrees Celsius, with the oxime being added dropwise (dissolved in anhydrous ether). The reaction was stirred under reflux for 10 hours. Careful addition of water and moist ether hydrolyzed the excess hydride, and the resulting white precipitate was separated by filtration. The ethereal filtrate was dried over sodium sulphate, resulting in a yellow solid.

The third step of the synthesis was as described in the non-deuterated synthetic paper: the d5-2-hydroxy-2-phenylethylamine was dissolved in THF with excess carbon-disulfide and catalytic pyridine. The resultant mixture was refluxed at 70 degrees Celsius for sixteen hours and the progress monitored via Thin-Layer Chromatography. Once the reaction was completed, the solution was cooled and concentrated via rotovap, washed in 1N hydrochloric acid, and extracted with DCM. This organic layer was dried with sodium sulfate, and the yellow solid product was recrystallized twice, yielding a 99% pure substance via combustion analysis.

All steps of the synthetic pathway were verified via mass spectroscopy as well as proton nuclear mass resonance spectroscopy. The final product was characterized by these methods and combustion analysis, and is available, along with its certificate of analysis.

Conclusion: A deuterated reference standard of the plant product barbarin is now available commercially, offering a vital point of interest for use in testing facilities and for determining appropriate International Residue Limits or Screening Limits of Detection for it and its metabolites.

Citation: Machin, J., Childers, T., Kudrimoti, S., Eisenberg, R., Fenger, C., Maylin, G., & Tobin, T. (2020) Synthesis and Characterization of d5-Barbarin for use in Barbarin Related Research. *Submitted to Journal of Analytical Toxicology*.

Personal Contribution: Development of multiple potential synthetic schema (including final one used), bench/wet chemistry, confirmation of structure via proton NMR, wrote manuscript

SYNTHESIS AND CHARACTERIZATION OF D5-BARBARIN FOR USE IN BARBARIN RELATED RESEARCH

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ABSTRACT:

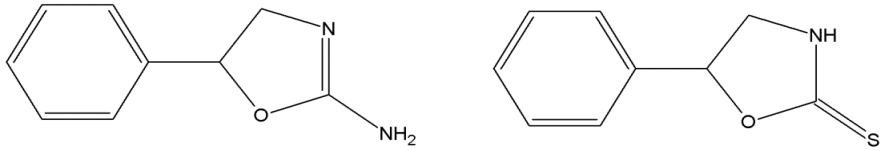
Based on structural similarities and equine administration experiments, Barbarin, 5-Phenyl-2-oxazolidinethione from Brassicaceae plants is a possible source of equine urinary identifications of aminorex, (RS)-5-Phenyl-4,5-dihydro-1,3-oxazol-2-amine, an amphetamine related US Drug Enforcement Administration (DEA) substance considered illegal in sport horses. We now report synthesis and certification of d5-barbarin to facilitate research on the relationship between plant barbarin and such aminorex identifications. D5-barbarin synthesis commenced with production of d5-oxime from d5-Acetophenone via butylnitrite in an ethoxide/ethanol solution. This oxime was then reduced with Lithium Aluminum Hydride to produce the corresponding alcohol-amine. Final ring closure of the alcohol amine was performed by addition of carbon disulfide with pyridine catalyst. The reaction product was purified by recrystallization and presents a stable white crystalline powder. This material was shown to be d5-barbarin by proton NMR, Mass Spectrometry, and Elemental Analysis and was purified and chemically characterized as 97% pure d5-barbarin for use as an Internal Standard in barbarin related equine forensic research.

KEYWORDS: Synthesis, d5-Barbarin, Internal Standard, Aminorex, Equine Forensic Science.

INTRODUCTION:

Based on structural similarities and equine administration experiments, barbarin, 5-Phenyl-2-oxazolidinethione from Brassicaceae plants is a possible source of aminorex (RS)-5-Phenyl-4,5-dihydro-1,3-oxazol-2-amine, (Figure 1) identifications in race and sport horse urines. Aminorex is an amphetamine related (Hofmaier, et al, 2013) US Drug Enforcement Administration (DEA) substance that is considered illegal in racing and sport horses. Aminorex is also an Association of Racing Commissioners International [ARCI] Class 1, Penalty class A foreign substance, so identifications of aminorex in equine samples can give rise to significant penalties for horsemen (ARCI Uniform Classification Guidelines for Foreign Substances January 2018 (V.13.4), the suggested penalties being in the order of a 1-year suspension and a $10,000 fine, as has happened at times in US horse racing (Whitmore 2017).

Figure 2.1.3.1 Structures of Barbarin and Aminorex



Aminorex Barbarin

Figure 1. STRUCTURES OF BARBARIN AND AMINOREX

This possible relationship between plant barbarin and equine urinary aminorex identifications was first suggested by Teale and Biddle, 2018, who had identified aminorex in English sport horse urines with no known exposure to aminorex or Levamisole, Levamisole being an equine anthelmintic and immune stimulant medication known to metabolize to aminorex (Barker, S A, 2009, Eiden et al., 2015). Reviewing their aminorex identifications: the absence of any known sources of Aminorex; the presence of a number of small plant related molecules in their equine urine samples; and the lack of presence of pemoline or rexamino, known metabolites of levamisole (Gutierrez, et al., 2010, Ho, et al, 2009); Teale and Biddle proposed that the likely source of their aminorex identifications was glucobarbarin, a barbarin precursor found in Brassicaceae plants.

Plants of the genus Barbarea, Brassicaceae family, contain glucobarbarin, a barbarin precursor. In these plants damage to the plant structure triggers hydrolysis of glucobarbarin by myrosinase to an intermediate which spontaneously cyclizes to barbarin, Figure 2, which functions as an insect repellant or attractant when the plant structure is damaged (Kjaer et. al., 1957). As set forth above, barbarin is related structurally to aminorex, and consumption of Brassicaceae plant fragments in equine feed is therefore a possible source of such unexplained aminorex identifications, as has been demonstrated in our recently published research (Maylin et al., 2019).

Figure 2.1.3.2 Natural Synthesis of Barbarin from Glucobarbarin

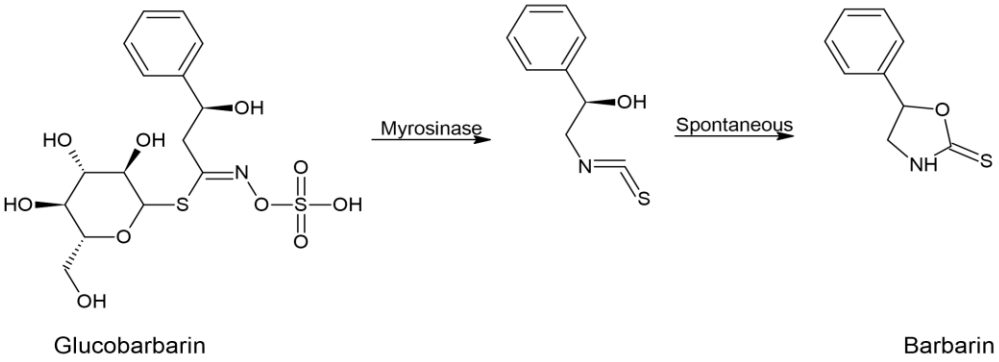


Figure 2: In plants, glucobarbarin is hydrolyzed by the enzyme myrosinase to the intermediate, above center, which spontaneously cyclizes to barbarin.

While this equine administration research (Maylin et al., 2019) links consumption of the Brassicaceae plant Brassica vulgaris to urinary aminorex identifications, it does not unequivocally identify barbarin as the proximate chemical source of these identifications. To address this matter, we therefore now report the synthesis, purification and characterization of d5-barbarin, the availability of which will allow more definitive identification of the relationship between plant barbarin, and equine consumption of such plant material being associated with equine urinary aminorex identifications.

DISCUSSION:

We have synthesized d5-barbarin for use in barbarin related research, specifically to enable more detailed evaluation of the relationship between equine/herbivore consumption of Brassicaceae plants containing glucobarbarin and urinary aminorex identifications. D5-barbarin was synthesized by a variant of our previously described barbarin synthesis method (Machin et al, 2018, Machin et al, 2020), as follows.

D5-barbarin synthesis commenced with production of d5-oxime from d5-acetophenone via butylnitrite in an ethoxide/ethanol solution, as described by Norman et al 1962. This oxime product was obtained in good yield and next reduced with Lithium Aluminum Hydride (Walter, 1952) to produce the corresponding alcohol-amine, again in good yield. Final ring closure of the alcohol amine was performed by addition of carbon disulfide with a pyridine catalyst, as described in our earlier barbarin synthesis communications (Machin et al, 2018, Machin et al, 2020). The final d5-barbarin reaction product presented as a stable white crystalline powder, was produced in good yield, purified by recrystallization and chemically characterized as d5-barbarin by proton NMR, Combustion Analysis and Mass Spectrometry and prepared for use as an Internal Standard in barbarin related research.

The research need for d5-barbarin comes from the apparent ability of Brassicaceae plant consumption by horses to result in low concentration urinary identifications of aminorex. Aminorex being an amphetamine related central nervous stimulant and a US DEA schedule 1 substance that is prohibited in racing and sport horses. A previous and unexpected source of aminorex identifications was Levamisole, a veterinary anthelmintic and immune stimulant at one time not infrequently prescribed in horses including racing horses. Identification of Levamisole administration as a source of aminorex identifications led to a marked reduction in the number of aminorex identifications in racing horses, but not to their complete elimination, as noted by Whitmore in 2017 and by Teale and Biddle in 2018.

Reviewing their unexpected aminorex identifications, Teale and Biddle noted that a number of small nitrogenous compounds were found in the samples, which compounds

pointed to plant origins for their aminorex identifications. Additionally, Teale and Biddle reported identification of aminorex itself in sample from some unidentified plants and noted that to their knowledge this was the first reported identification of aminorex in a plant. Reviewing these unexpected findings, Teale and Biddle noted that research was required to determine the structure of plant derived analytes that might serve as markers of plant ingestion and therefore as markers of botanical origins for aminorex identifications in equine urine samples.

The major conclusion of the Teale and Biddle findings was that plants of the Brassicaceae family contain glucobarbarin which may serve as a plant precursor of barbarin, with plant barbarin itself usually serving as an aversive substance to discourage herbivory. Addressing this problem, we first synthesized barbarin as a reference standard for barbarin and aminorex related research. As this synthesis was in progress in Spring 2018, we observed Kentucky Brassica vulgaris, colloquially Kentucky "Yellow Rocket" in flower in Kentucky. We therefore definitively identified, harvested, cleaned and dried these plants and sent them to Professor Maylin in New York for equine administration experiments. The outcome of these experiments was that four hours after consuming barbarin the experimental horses produced aminorex containing urines, linking consumption of Kentucky Brassica Vulgaris, colloquially "Yellow Rocket", to urinary aminorex identifications.

At this time, however, it is unclear as to what precisely is the proximate chemical source of these post Brassica vulgaris administration urinary aminorex identifications. Whatever the mechanism, however, reviewing a number of apparently low concentrations aminorex identifications in post-race samples, the Kentucky Horse Racing Commission chose to rescind a "positive" identification at their April 17th, meeting in Lexington, based apparently on uncertainties concerning the origins of the identification (Voss, 2018). In particular, it appears that as well as aminorex, barbarin was identified in the samples from the horse, giving rise toe possibility that the aminorex identification was due to inadvertent exposure of the horse to a plant source of barbarin, as we will now detail.

Our initial working hypothesis was that barbarin formed in the Brassicaceae plant associated with harvesting damage to the plant was the likely proximate source of aminorex, as set forth in Figure 2. In this model barbarin is present in the plant on ingestion and is metabolically transformed in/by the horse to aminorex which is then excreted in the urine, leading to urinary aminorex identifications. In this model the metabolic transformation from barbarin to aminorex occurs in the horse and is driven by the metabolic capabilities of the horse (Tobin, 1981). An alternative hypothesis is that the barbarin is excreted in the urine and that the transformation from barbarin to aminorex occurs in the urine sample, although this seems a somewhat less likely possibility ((Soma, et.al 2008).

The fact that aminorex consumption of Brassicaceae plants is associated with aminorex identifications in equine urine has important scientific and forensic implications. aminorex has been identified in equine urine samples without evidence of exposure to Levamisole. Between the years of 2016 and 2018, 11 unexplained low level aminorex identifications have been reported in US racing horses: 3 in Illinois between 2016 and 2017, 5 in Massachusetts in 2017, 3 more in Kentucky in 2018, and 1 more in Kentucky in 2019 (Holloway, 2019). A proposed source of such urinary aminorex identifications is an equine driven metabolic transformation of barbarin or a related substance from plants of the Brassicaceae family inadvertently entering equine feedstuffs (Teale and Biddle, 2018) to aminorex, as has previously been shown to occur following Levamisole administration. Experimental approaches to this question were hindered by the lack of availability of chemically pure barbarin and an appropriate internal standard, i.e., d5-barbarin for use both as research and internal standards in botanical and equine related research.

A further consideration with respect to these aminorex identifications is that given the worldwide distribution of Brassicaceae plants and related glucobarbarin containing plants, aminorex appears to meet the definition of a plant related environmental substance appearing in post-event urine samples. As such, these aminorex identifications are in many ways equivalent to the Scopolamine identifications that have been sporadically reported identified in competition horses worldwide for now thirty or more years, as we reported a number of years ago (Brewer et al, 2014).

The most widely used approach to this trace level identification problem is that adopted by the International Federation of Horseracing Authorities (IFHA): namely the identification of an International Residue Limit or Screening Limit of Detection [SLOD] below which defined plasma or urinary concentration the identification is not reported for regulatory action. This approach is set forth in the IFHA website document on this matter and as has also recently been communicated with reference to trace level identifications of Methamphetamine (Brewer et al 2016) and Naproxen (Machin et al, 2019) identifications in equine post-race samples.

In closing, d5-barbarin has now been synthesized, purified and characterized. D5-barbarin presents as a stable white crystalline substance and is now available as a stable isotope internal standard for analytical, forensic, or toxicological research and, if required, in larger quantities such as may be required for other experimental purposes (Maylin et al 2018, Tobin, 1981).

EXPERIMENTAL:

Synthesis of d5-barbarin was a three-step process, The final step of ring closure to get the product i.e., d5-barbarin was based on previous report on the synthesis of the required substances, unlabeled barbarin (Norman et al, 1962, Walter 1952, Santoro et al,1976) and personal communications with Dr. Richard A. Sams, as follows:

Figure 2.1.3.3 Synthesis of d5-Barbarin from d5-Acetophenone

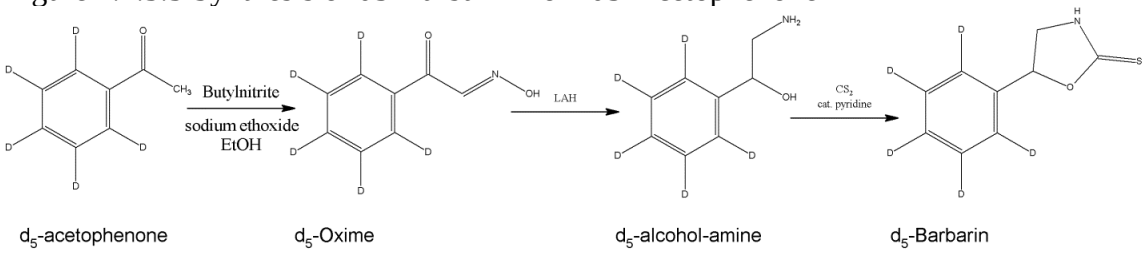


Figure 3: Synthesis of d5-barbarin, started with d5-acetophenone, butylnitrite and sodium ethoxide, yielding d5-oxime, then reduced by lithium aluminum hydride, yielding d5-

alcohol-amine, then reacted with carbon disulfide with catalytic pyridine to yield d5-barbarin.

Step 1: Synthesis of d5-oxime from d5-acetophenone, as in the synthesis scheme in Figure 3 above. Step 1 commenced when Butyl nitrite (0.96 ml, 1 mole) was taken in ice-cold ethanol (50 ml) and to it was added sodium ethoxide (0.565 gm, 1 mole). To this solution d5-acetophenone (1 gm, 1 mole) dissolved in 10 ml of ethanol was added dropwise over 30 minutes, the solution being maintained ice cold until the addition of d5-acetophenone was complete and the solution then stirred until it reached room temperature. Stirring then continued for 3 hr., following which the solution was held overnight. The following day the formed precipitate was filtered and washed with ether. The washed precipitate was dissolved in a minimum quantity of water, acidified with glacial acetic acid and the resulting off white solid filtered and recrystallized with ethanol. The estimated yield from this reaction was 45%, and the recovered d5-oxime material was characterized by 1H NMR and Mass Spectrometry as appropriate for d5-oxime, m/z 154.0791, H1 NMR(DMSO) in ppm: 1H (S, 8.0, 1H), as in Figure 4, below.

Figure 2.1.3.4 1H-NMR of recovered d5-Oxime

Fig 4

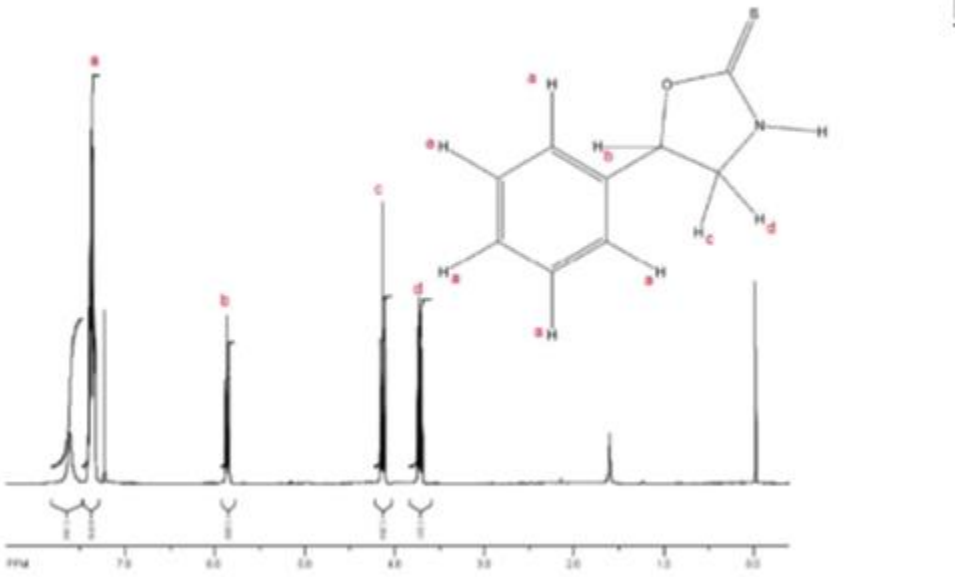


Figure 4: The recovered d5-oxime material as characterized by 1H NMR

Step 2: Synthesis of the d5-alcohol-amine: 0.115gm (4eq) LiAlH4 is taken in round bottomed flask under argon. The flask was then fitted with a dropping funnel and to it was added 30 ml of anhydrous ether under argon and the LIAlH4/ether slurry was stirred at 0oc. d5-oxime (0.117 gm, 1eq), dissolved in anhydrous ether(10ml) was added dropwise, and the mixture was stirred and refluxed for 10 hr. Careful addition of water and moist ether hydrolyzed the excess hydride, and the resulting voluminous white precipitate was separated by filtration and the ethereal filtrate dried over sodium sulfate. After removal of the solvent, the residue yielded a yellowish solid (0.075gm), with an overall reaction yield of about 70%. The resultant material was characterized as d5-alcohol-amine by NMR 1H NMR (CDCl3, 400 MHz) δ (ppm): 4.87 (t,1H1), 3.10(dd, 1H2) and 2.85(dd,1H3), 4.80(1H, OH), 1.98(2H, NH2) Figure 5, below, and by Mass spectrometry, m/z 142.115

Figure 2.1.3.5 Mass Spectral Analysis of d5-alcohol-amine

Fig 5

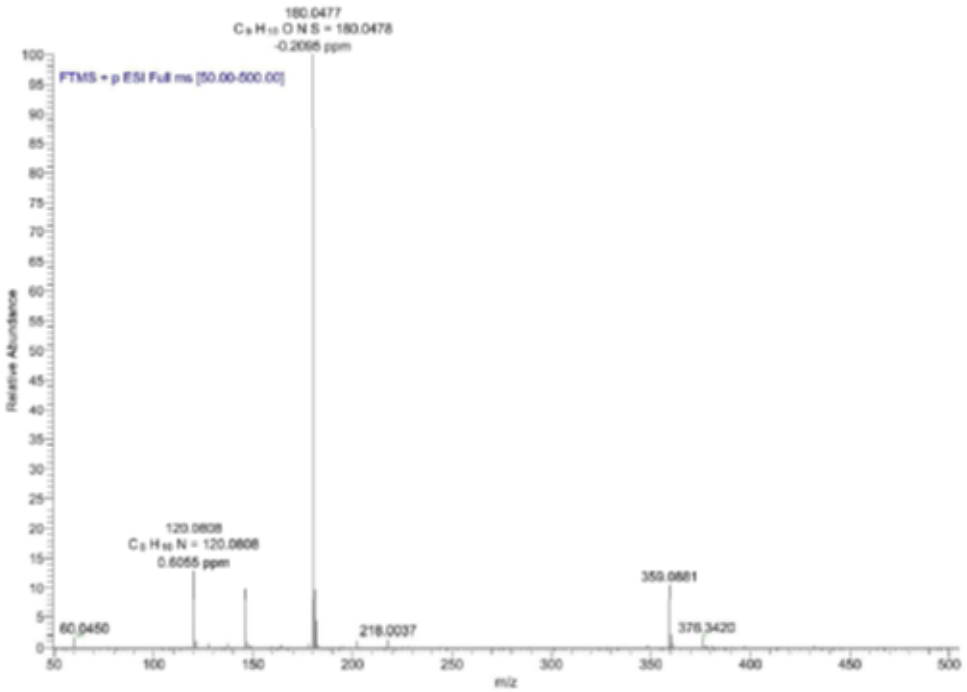


Figure 5: The resultant material was characterized as d5-alcohol-amine by mass spectroscopy.

Step 3: Synthesis of d5-barbarin: To the d5-2-hydroxy-2-phenylethylamine (d5-alcohol-amine) (0.075 gm, 1 eq) in THF (20 ml) was added excess carbon-disulfide (1ml) in the presence of 1 equivalent of pyridine. The resultant mixture was refluxed at 70°C for 16hrs and the progress of the reaction monitored by TLC. Once the reaction was complete, the system was cooled and the reaction mixture concentrated on a rotovap, washed with 1N HCl, water and the aqueous layer extracted with DCM. The resulting organic layers were combined, dried with sodium sulfate and the yellow solid product recrystallized twice using a DCM/Hexane solvent system. The final recrystallized product was 99% pure by Combustion/Elemental Analysis and its chemical structure was confirmed as d5-barbarin by proton NMR 1H NMR (CDCl3, 400 MHz) δ (ppm): 5.85 (t, 1H), 3.71(dd, 1H) and 4.11(dd, 1H), (Figure 6), and by Mass Spectrometry, (m/z): [M+H] + for C9H4D5NOS 185.0719, (Figure 7).

Figure 2.1.3.6 1H NMR of d5-Barbarin Product

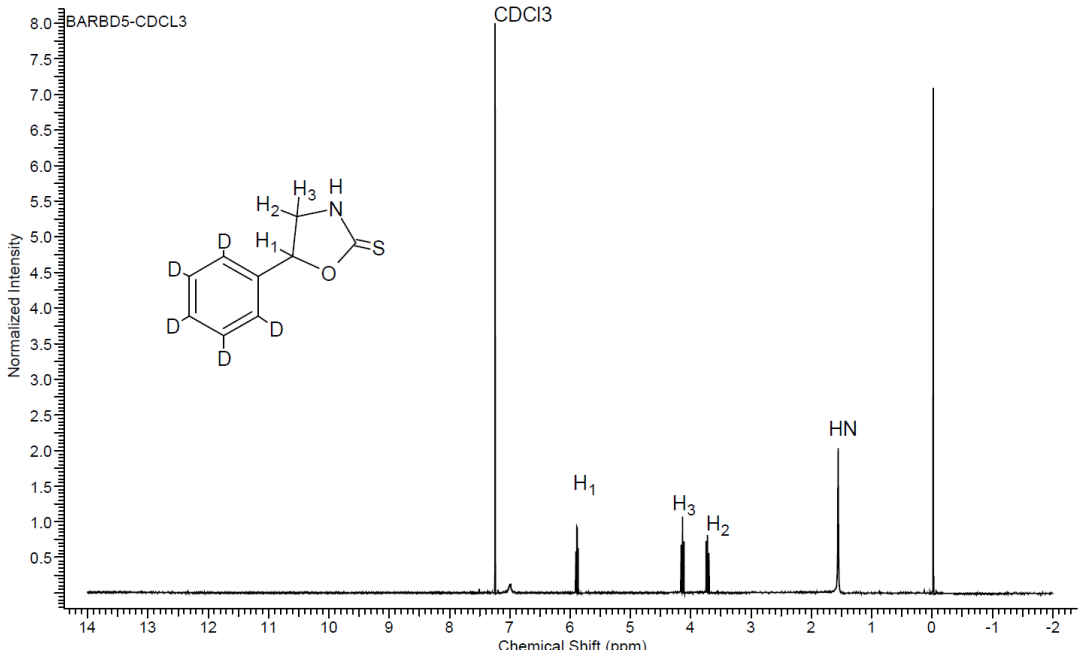


Figure 6 The resultant material was confirmed as d5-barbarin and by proton NMR 1H NMR.

Figure 2.1.3.7 Mass Spectrum of Resultant d5-Barbarin

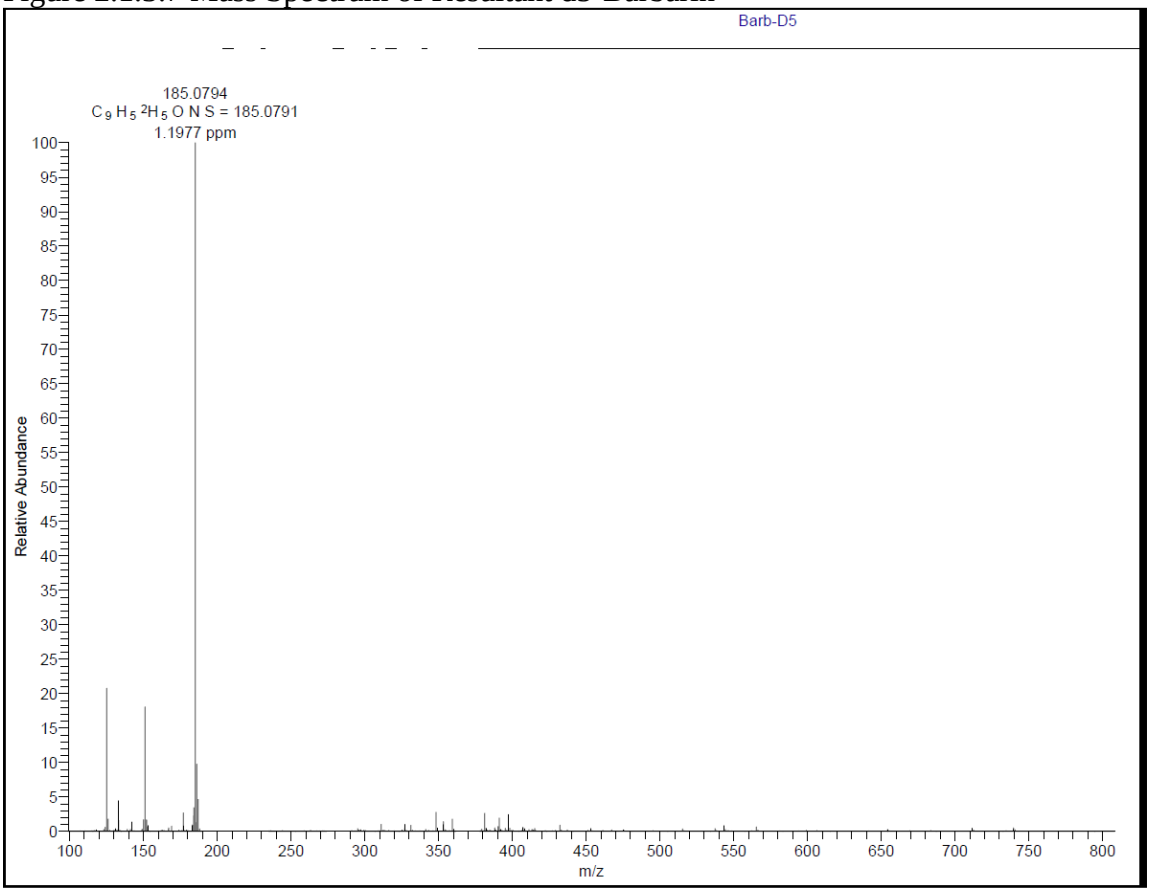


Figure 7 The resultant material was also confirmed as d5-barbarin by Mass Spectrometry [M+H] + for C9H4D5NOS 185.0719, as above.

RESULTS:

The white crystalline material obtained following purification was characterized as follows: Analysis by proton Nuclear Mass Resonance yielded the following, 1H-NMR, (CDCl3,400 MHz) δ (ppm): 5.85 (t, 1H), 3.71(dd, 1H) and 4.11(dd, 1H). and 1.60(1H, NH) as shown in Figure 6 above. Elemental analysis resulted in CHNS, Calc: C, 58.66%, H, 5.06%, N 7.60%, S, 17.40%. Found: C 57.31%, H 5.24%, N 7.18%, S 16.40%; indicating 97% purity. High Resolution Mass Spectrometry presented the following: and mass spectrometry, (m/z): [M+H] + for C9H4D5NOS 185.0719, as in Figure 7 above. Based on NMR, mass and elemental analysis data we have characterized the final product as d5-barbarin for use in barbarin related research.

ACKNOWLEDGEMENTS:

This research was made possible by research support from The Equine Health and Welfare Alliance, Inc, Versailles, Kentucky, and the United States Trotting Association, Columbus, OH. Further support came from the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch Program under project KY014051 Accession Number 1010609 and also by grants from the USDA Agriculture Research Service Specific Cooperative Agreement #58-6401-2-0025 for Forage-Animal Production Research, the Kentucky Department of Agriculture and the Kentucky Thoroughbred Association Foundation and by support for the Kentucky Agricultural Experiment Station as provided by the National Institute of Food and Agriculture (NIFA) and the Commonwealth of Kentucky. Other support includes research support from The National Horsemen's Benevolent and Protective Association and the Alabama, Arizona, Arkansas, Ontario, Canada; Charles Town, WV; Florida, Indiana, Iowa, Kentucky, Louisiana, Michigan, Minnesota, Nebraska, Ohio, Oklahoma, Oregon, Pennsylvania, Tampa Bay Downs, Florida, Texas, Washington State, and West Virginia Horsemen's Benevolent and Protective Associations. Published as paper #502 from T Tobin and the Equine Pharmacology, Therapeutics and Toxicology Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky.

LITERATURE CITED:

(1) Hofmaier, T., Luf, A., Seddik, A., Stockner, T., Holy, M., Freissmuth, M., Ecker GF, Schmid R, Site HH, & Kudlacek, O. (2014). Aminorex, a metabolite of the cocaine adulterant levamisole, exerts amphetamine like actions at monoamine transporters. Neurochemistry international, 73, 32-41.

(2) ARCI Uniform Classification Guidelines for Foreign Substances January 2018 (V.13.4).

(3) Whitmore, Dennis, 2017 Horseplop.com, Harness Racing, Aminorex, November 28th, 2017.

(4) Teale, P. and Biddle, S. (2018). Possible Plant Sources of Aminorex and Potential markers of Their Ingestion in the Horse. Proceeds of the 22nd International Conference of Racing Analysists and Veterinarians. Dubai, UAE in press.

(5) Barker, S. A. (2009). The formation of Aminorex in racehorses following levamisole administration. A quantitative and chiral analysis following synthetic Aminorex or levamisole administration vs. Aminorex-positive samples from the field: a preliminary report. Journal of veterinary pharmacology and therapeutics, 32(2), 160-166.

(6) Eiden, C., Peyrière, H., Diot, C., & Mathieu, O. (2015). Prevalence of levamisole and Aminorex in patients tested positive for cocaine in a French University Hospital. Clinical toxicology, 53(7),604-608

(7) Gutierrez, J., Eisenberg, R. L., Koval, N. J., Armstrong, E. R., Tharappel, J., Hughes, C. G., &Tobin, T. (2010). Pemoline and Tetramisole 'positives' in English racehorses following levamisole administration. Irish veterinary journal, 63(8), 498.

(8) Ho, E. N. M., Leung, D. K. K., Leung, G. N. W., Wan, T. S. M., Wong, A. S. Y., Wong, CH. Soma LR, Rudy JA, Uboh C, & Sams, R. (2009). Aminorex and rexamino as metabolites of levamisole in the horse. Analytica chimica acta, 638(1), 58-68.

(9) Kjaer, A., & Gmelin, R. (1957). IsoThiocyanates. XXVII. A New isoThyiocyanate Glucoside (GlucoBarbarin) Furnishing (-)-5-Phenyl-2-oxazolidinethione upon Enzymic Hydrolysis. L. Acta Chemica Scandinavica, 11, 906-907. doi: 10.3891/acta.chem.scand.11-0906

(10) Maylin, G., Fenger, C., Machin, J., Kudrimoti, S., Eisenberg, R., Green, J., & Tobin, T. (2019). Aminorex identified in horse urine following consumption of Barbarea vulgaris, a preliminary report. Irish Veterinary Journal, 72(1), 15. p2-8.

(11) Norman, JJ, Heggie, RM, and Larose JB, 1962 Oximes: 1. The synthesis of some substituted 2-oximinoacetophenones Canadian Journal of Chemistry, 40(8): 1547-1553, https://doi.org/10.1139/v62-233

(12) Walter, Jr, C R 1952 Preparation of Primary Amines by Reduction of Oximes with Lithium Aluminum Hydride and by the Leuckart Reaction, Journal of American Chemical Society,74(20),5185-5187.

(13) Santoro, R., Warren, R., & Roberts, G. (1976). Spontaneous formation of 4-methyl-5-phenyloxazolidine-2-thione from phenylpropanolamine. Journal of Chromatography A, 117(2),375-382.

(14) Machin, J. Kudrimoti, S. Eisenberg, R., Fenger, C., Hartmann, P., Maylin, G., Tobin, T., and Shults, T. (2018). "Synthesis, Characterization and Certification of Barbarin, a Possible Botanical Source of Aminorex Identifications". Submitted for presentation and publication, the Society of Forensic Toxicology (SOFT) Annual Meeting, Oct 07-12, 2018. Minneapolis, Minnesota, p 92.

(15) Machin 2020 reference---Need the complete Ref

(16) Voss N, (2018) https://www.paulickreport.com/news/the-biz/the-trouble-with-aminorex/ April 17, 2018. Accessed March 21, 2020

(17) Tobin, T. and Charles C. Thomas, (1981). Drugs and the performance horse. Springfield Illinois,1981.

(18) Soma, L.R., Rudy, J.A., Uboh, C.E., XuF.; Snapp, H.M. (2008) Pharmacokinetics and effects of aminorex in horses. American journal of veterinary research,2008;69(5):675-681.

(19) Holloway, K, (2019) Aminorex and Pemoline Identifications in US Racing: Personal communication from the Association of Racing Commissioners International, 2/20/2019.

(20) Brewer K1, Dirikolu L2, Hughes CG3, Tobin T4. (2014) Scopolamine in racing horses: trace identifications associated with dietary or environmental exposure. Vet J. 2014 Mar;199(3):324-31

(21) Brewer, K., Shults, T. F., Machin, J., Kudrimoti, S., Eisenberg, R. L., Hartman, P, and Tobin, T. (2016). A cluster of trace-concentration methamphetamine identifications in racehorses associated with a methamphetamine-contaminated horse trailer: A report and analysis. The Canadian Veterinary Journal, 2016,57(8), 860-864.

(22) Machin, J. K. Brewer, M. Catignani T.F. Shults C. Fenger G.A. Maylin and T. Tobin (2019) An interim screening limit of detection for naproxen in equine plasma: a review and analysis; Comparative Exercise Physiology,2019,16,153-160.

2.2 Trace Level Therapeutic Medication Overages/Positives

2.2.1 A Pilot Pharmacokinetc / Withdrawal Time Study Using a Full Clinical Dose of Mepivacaine in Thoroughbred Horses

Abstract: Mepivacaine is a widely used local anesthetic and generally approved therapeutic medication in performance horses. To guide the racing community with respect to its therapeutic use close to regulated events, standard dosing regimens and withdrawal time guidelines associated with defined regulatory thresholds are required. The Association of Racing Commissioners International [ARCI] schedule recommends a 72-hour withdrawal after a dose of 0.07mg/kg, about 35.0 mg/horse, administered subcutaneously [SQ] with a regulatory threshold of 10 ng/mL of total 3-hydroxymepivacaine in urine. At times higher doses of mepivacaine may be clinically

required, and the goal of this study was to provide guidance for a 400 mg SQ dose. This dose was administered to 6 horses, and the urinary concentrations of total 3-hydroxymepivacaine determined by liquid chromatography/tandem mass spectrometry for out to 5 days. Urinary concentrations of total 3-hydroxymepivacaine exceeded the 10 ng/mL urinary threshold in all samples collected, including on day 5. Based on these data and previous published research, we created a three-compartment model of the urinary concentrations of total 3-hydroxymepivacaine and calculated withdrawal time estimates following administration of 35 to 400 mg doses of mepivacaine to Thoroughbred horses. These data provide best withdrawal time guidelines for veterinarians treating Thoroughbreds horses with between 35 and 400 mg SQ doses of mepivacaine, including our best calculated projections of withdrawal times out to seven days post administration.

Introduction: Current testing recommendations by the RMTC for mepivacaine, a commonly used local anesthetic in the equine industry, are 10.0 nanograms total hydroxymepivacaine per milliliter of urine or the Limit of Detection of mepivacaine in plasma/serum. These values are associated with a seventy-two-hour withdrawal time for a dose of 0.07 mg/kg (approximately 35 mg total) via subcutaneous injection. However, standard doses in practice often reach up to 400 mg total injected. For such a higher dose,

no guidance is provided, and horsemen and veterinarians are left to their own devices for determining when is appropriate to enter their horses. With that in mind, a pharmacokinetic study was performed to give guidance for a full clinical dose of mepivacaine.

Experimental: Six horses were administered 400mg of mepivacaine subcutaneously via single injection into the left shoulder. Pre-injection urine samples were collected and stored. Likewise, urine samples were collected within 2 hours of the injection time each day thereafter for five days. Samples were frozen and analyzed via mass spectrometry for urinary 3-hydroxymepivacaine. Results were analyzed via pharmacokinetic analysis and a three-compartment model with an absorption compartment was found to be in best agreement with the values observed.

The pharmacokinetic parameters determined appear to be in good agreement with other studies on mepivacaine in horses, though due to the testing facilities much lower limit of detection, a much longer terminal-half-life was observed compared to what was previously reported, an important detail for determining withdrawal guidelines. The largest variance between the model and the mean plasma concentrations occurred at 24 hours (the model being approximately 50% of the observed values) but quickly converges with the observed data, and at five days is within 6% of the same value. At all time-points, it is within one standard deviation of the observed datapoints mean.

Of interest, the relative standard deviation of the population appears to change linearly with time, indicating that injection site differences or similar likely play a major role in the early variance in plasma concentrations.

Using said trend in the relative standard deviation, a withdrawal guideline chart was developed to guide veterinarians and horsepersons when a dose is most likely to be acceptable within a certain amount of time of a race. As such, the 95/95 tolerance interval for a full clinical dose of 400mg is likely to clear the horse at 134 hours (approximately 5.5 days) for a threshold of 10ng/mL.

Conclusion: Based on the reported data and the lipophilicity of mepivacaine, the authors recommend the following withdrawal guidelines for varying doses: 96 hours for 100mg dose, 120 hours for a 200mg dose, and 168 hours for a 400mg dose.

Citation: Machin, J., Maylin, G., Fenger, C., Hunt, R., Bladon, K., Sacopulos, O., & Tobin, T. (2018) A Pilot Pharmacokinetic/Withdrawal Time Study Using a clinical Dose of Mepivacaine in Thoroughbred Horses. *Submitted to International Conference of Racing Analysts and Chemists*.

Personal Contribution: Assisted veterinarian in administration of mepivacaine; collected urine and blood samples, processed samples for storage and shipping, shipped samples to associated forensic laboratory, performed pharmacokinetic analysis, developed pharmacokinetic and descriptive models, performed statistical analyses as published, wrote manuscript

A PILOT PHARMACOKINETIC / WITHDRAWAL TIME STUDY USING A CLINICAL DOSE OF MEPIVACAINE IN THOROUGHBRED HORSES

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ABSTRACT

Mepivacaine is a widely used local anesthetic and generally approved therapeutic medication in performance horses. To guide the racing community with respect to its therapeutic use close to regulated events, standard dosing regimens and withdrawal time guidelines associated with defined regulatory thresholds are required. The Association of Racing Commissioners International [ARCI] schedule recommends a 72-hour withdrawal after a dose of 0.07mg/kg, about 35.0 mg/horse, administered subcutaneously [SQ] with a regulatory threshold of 10 ng/mL of total 3-hydroxymepivacaine in urine. At times higher doses of mepivacaine may be clinically required, and the goal of this study was to provide guidance for a 400 mg SQ dose. This dose was administered to 6 horses, and the urinary concentrations of total 3-hydroxymepivacaine determined by liquid chromatography/tandem mass spectrometry for out to 5 days. Urinary concentrations of total 3-hydroxymepivacaine exceeded the 10 ng/mL urinary threshold in all samples collected, including on day 5. Based on these data and previous published research, we created a three-compartment model of the urinary concentrations of total 3-hydroxymepivacaine and calculated withdrawal time estimates following administration of 35 to 400 mg doses of mepivacaine to Thoroughbred horses. These data provide best withdrawal time guidelines for veterinarians treating Thoroughbreds horses with between

35 and 400 mg SQ doses of mepivacaine, including our best calculated projections of withdrawal times out to 7 days post administration.

KEYWORDS: Mepivacaine; Withdrawal Times; Drug-Testing; Horseracing; Thoroughbred

AIM

The objective of this study was to provide guidance for practitioners administering the therapeutic medication mepivacaine (Carbocyanine/Polocaine) (Tobin, 1981; Tobin 2013), Figure 1, at doses higher than the 0.07mg/kg (35 mg) subcutaneous (SQ) dose specified in the Association of Racing Commissioners International [ARCI] withdrawal guideline. Current ARCI guidelines recommend withdrawal at least 72 hours prior to testing after a SQ dose less than 0.07mg/kg. The ARCI threshold is 10 ng/mL in urine of "total 3-hydroxymepivacaine" (RMTC website) and below the Limit of Detection (LOD) in plasma (ARCI website), although the Racing Medication and Testing Consortium [RMTC] has recently recommended a 50 pg/mL plasma/serum "cut-off" for parent mepivacaine (Personal Communication, S.A. Barker).

Figure 2.2.1.1 Structure of Mepivacaine and its Metabolites of Interest

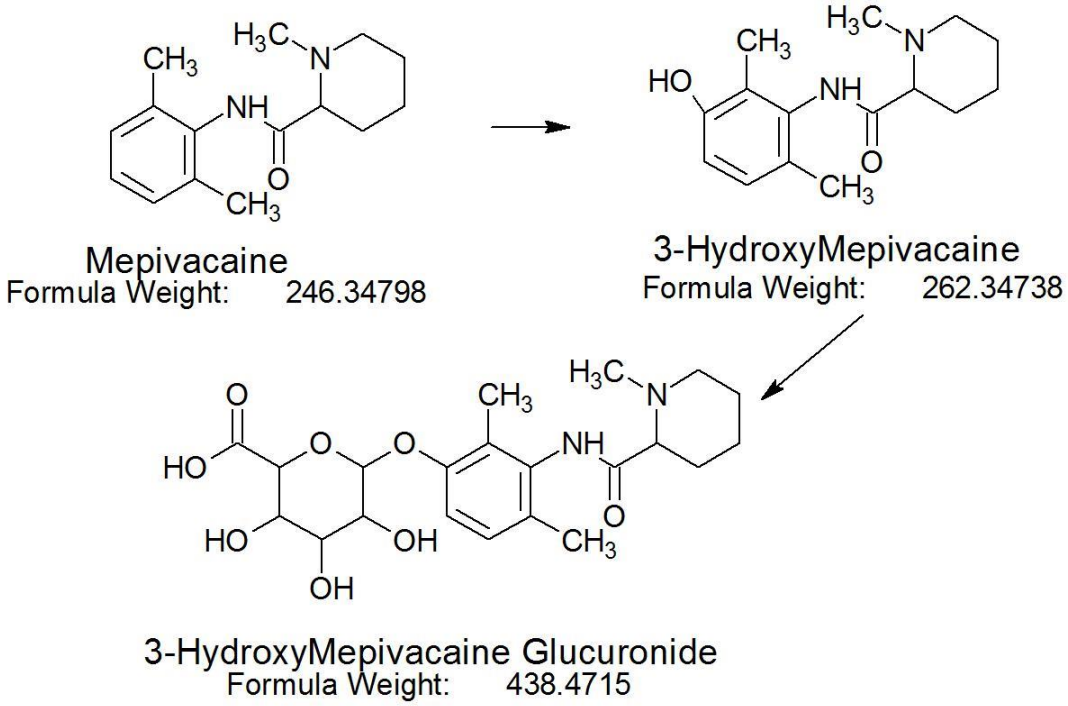

Figure 1: Structure of Mepivacaine and its Metabolites of Interest *showing the chemical structures and formula weights for mepivacaine, its Phase 1 metabolite, 3-hydroxymepivacaine, and its major urinary metabolite 3-hydroxymepivacaine glucuronide.*

As noted by Toutain (2010), “one of the most difficult tasks for a veterinarian when treating a competition horse is to decide a withdrawal time.” A withdrawal time is a recommendation by a veterinarian as when to stop treating/last administer a medication prior to a regulated event. The veterinarian reviews regulatory literature, taking care to distinguish between European “Detection Times” data and US “Withdrawal Time” data, reviews dose, data and the number of experimental animals used and other relevant scientific literature. He/she also reviews the clinical data associated with the administration, including formulation, route of administration, dosage regimen, duration of treatment and sources of animal-related variability, such as age, sex, breed, and training/racing history. The veterinarian then estimates a withdrawal time, knowing that “the main sources of uncertainty are of biological origin and cannot be reduced by managerial options” (Toutain, 2010).

This study reports a pilot pharmacokinetic evaluation of a 400 mg subcutaneous dose of mepivacaine to horses with the goal of guiding veterinarians defining withdrawal time guidelines at doses of mepivacaine above the ARCI specified dosage. We selected a 400 mg subcutaneous dose of mepivacaine, the highest dose routinely required in veterinary practice as in, for example, a Caslick’s operation. This study focused on determining the final terminal urinary half-life of total 3-hydroxymepivacaine in urine with the goal of guiding veterinarians faced with clinical situations requiring a higher than 35 mg administration of mepivacaine.

MATERIALS AND METHODS:

Horses

Six horses owned by Equine Integrated Medicine, PLC were used in this study (age mean ± standard deviation, range 7 ± 5 years, 2 to 16 years, 1 colt, 3 geldings, 2 mares). Horses were determined to be healthy by physical examination by an experienced practitioner. Caslick’s procedures were performed on 2 mares using 400 mg of mepivacaine SQ (CARBOCAINE® V Sterile Aqueous Solution, 2% Mepivacaine hydrochloride, USP), provided courtesy of Zoetis (Parsippany, NJ). Colts and geldings received 400 mg mepivacaine SQ as a single injection over the left shoulder.

Overall study design

Pre-injection urine samples were collected from the six horses in this study and stored pre-drug administration. Horses were trained to urinate by whistling. Urine samples were collected by free catch within 2 hours of the injection time each day for five days. Samples were frozen and sent frozen overnight to the New York Drug Testing and Research Program, 777 Warren Rd., Ithaca, NY 14850, USA for quantitative analysis for total urinary 3-hydroxymepivacaine.

Urinary Hydroxymepivacaine concentration determination

Quantitative analysis for total urinary 3-hydroxymepivacaine was performed in the New York State Racing and Wagering Board Laboratory using their Racing Medication and Testing Consortium (RMTC) and ISO-17025 accredited method. The Limit of Detection of the method was 5 pg/mL in urine and the Lower Limit of Quantitation (LLOQ) of the method was 10 pg/mL of total 3-hydroxymepivacaine in urine.

The analytical reference standard for 3-hydroxymepivacaine was purchased as a stock solution of 0.102 mg/mL from Frontier BioPharm (Richmond, KY, USA). The analytical reference standard for mepivacaine-d3 (used as internal standard) was purchased from Toronto Research Chemicals (North York, Ontario, Canada), and prepared as a stock solution at 1.00 mg/mL in methanol. Acetonitrile, ethyl acetate, hexane, chloroform, and methanol were purchased from EMD Millipore (Billerica, MA, USA). Mepivacaine-d3 was used as it is the specified internal standard in the RMTC ISO-77025 approved procedure used for Mepivacaine regulation in New York State. A further consideration is that certified 3-hydroxymepivacaine-d3 of adequate purity was not available when the approved method was developed/validated. Methyl tert-butyl ether, ammonium acetate, and acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). β-glucuronidase, 1140,000 /ml of beta glucuronidase activity, 2,700 units/ml of sulfatase activity was purchased from Campbell Science (Logan, UT, USA). Deionized water was produced onsite to the specification of 18.2 mΩ. All reagents were of HPLC grade or better.

3-Hydroxymepivacaine working solution was prepared by dilution of the 0.102 mg/mL stock solution with methanol to a concentration of 5.0 ng/µL. Three calibrators were prepared by dilution of the working standard solution with drug-free urine to concentrations of 50, 500, and 1000 ng/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. One mL of urine was aliquoted

into new, labeled test tubes. To each tube, mepivacaine-d3 stock solution was added followed by the addition of 1.0 mL of 1 M ammonium acetate buffer, pH 4.4 and 1.0 mL of 10,000 IU/mL β-glucuronidase in water. The pH was verified, and each tube was loosely capped. The samples were incubated in a 65°C bath for 2 hours. The samples were cooled to room temperature and 2.0 mL of saturated carbonate buffer, 2.0 mL of carbonate buffer, pH 9.5, and 5.0 mL of chloroform were added. The samples were mixed by rotation for 10 min, centrifuged at 2400 rpm for 5 minutes. The upper aqueous layer was aspirated to waste. The chloroform layer was transferred to a clean, labeled test tube containing 5.0 mL of 1% acetic acid. 1.0 mL of hexane was added, and the samples were capped, mixed by rotation for 10 min, and centrifuged at 2400 rpm for 5 min. The acid layer was transferred to a clean, labeled test tube and reextracted with 4.0 mL of saturated carbonate buffer and 5.0 mL of chloroform. The samples were mixed by rotation for 10 min, centrifuged at 2400 rpm for 5 minutes. The upper organic layer was transferred to a tapered test tube and dried under nitrogen at 40°C. Samples were dissolved in 100 µL of 1:1:1 acetonitrile: methanol: DI water. 2.5 µL was injected into the LC-MS/MS system, Agilent 6400 series triple quadrupole mass spectrometer coupled with an HPLC chromatography system.

The concentration of total 3-hydroxymepivacaine in urine was measured by LC-MS/MS using positive electrospray ionization. The mass spectrometer was operated using Agilent's Jet Stream Technology (Agilent Technologies, Palo Alto, CA, USA). Chromatography employed a Zorbax SB-CN column (2.1 mm x 150 mm column, 1.8 µm particle size, 80Å) (Agilent Technologies) and a linear gradient of 50:50 acetonitrile:10 mM ammonium acetate in water (Mobile Phase A, MP A) and 45:45:10 acetonitrile: methanol: DI H2O (Mobile Phase B, MP B) with a constant flow rate of 0.4 mL/min. The initial concentration of MP B was held at 10% for 2.0 min, ramped to 95% over 1.5 min, and held at that concentration for 2.0 min before re-equilibrating for 0.5 min at the initial concentration.

Detection and quantification were performed using selective reaction monitoring (SRM) of the precursor ion for 3-hydroxymepivacaine (m/z 263) and the internal standard (m/z 250). The responses for the product ions for 3-hydroxymepivacaine and mepivacaine-d3, m/z 98 and 101, were plotted and peaks at the appropriate retention times were integrated using MassHunter software (Agilent) (Woods, et al, 2008). MassHunter

software was used to generate calibration curves from ion peak area ratios and quantitate 3-hydroxymepivacaine in all samples by non-weighted linear regression analysis.

Pharmacokinetic analysis

Concentration versus time evaluations for total 3-hydroxymepivacaine were tabulated and mean values were determined for each timepoint. Likewise, data from the 486 mg dose from the previous study by Harkins, et al (1999) were normalized to a dose of 400 mg by multiplying all reported concentrations by a factor of 400/486. The Method of Residuals was used to determine an equation to estimate the mean urine total 3-hydroxymepivacaine concentrations as a function of time after a 400 mg dose. This equation was then again dose-normalized to the 400 mg dose and checked against other concentrations (by dividing other SQ doses by 400) from Harkins, et al (1999) to test goodness of fit. The generalized equation for this model is shown in **Equation 1**

Equation 1

$$C_{urine,time} = \frac{X}{N}(Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t} - (A + B + C)e^{-k_a t})$$

Where, X = dose administered

N = normalization factor

A, B, C, α, β, γ, ka = constants derived from Method of Residuals

t = time post-administration

Using the constants derived from the Method of Residuals, it was then possible to model several pharmacokinetic rate constants by simultaneously solving **Equations 2, 3, & 4.**

Equation 2

$$A = \frac{1}{V} * \frac{k_a}{k_a - \alpha} * \frac{k_{21} - \alpha}{\alpha - \beta} * \frac{k_{31} - \alpha}{\alpha - \gamma}$$

Equation 3

$$B = \frac{1}{V} * \frac{k_a}{k_a - \beta} * \frac{k_{21} - \beta}{\beta - \alpha} * \frac{k_{31} - \beta}{\beta - \gamma}$$

Equation 4

$$C = \frac{1}{V} * \frac{k_a}{k_a - \gamma} * \frac{k_{21} - \gamma}{\gamma - \beta} * \frac{k_{31} - \gamma}{\gamma - \alpha}$$

In order to predict what the 95/95 tolerance interval's upper limit in a population for a given dose at a specific time post administration, the Relative Standard Deviation [RSD] was calculated at each timepoint, and an equation to estimate the RSD was determined. One horse (Max) was excluded from the calculation of the RSD as this horse showed substantial, unexplained individual variation in the drug absorption and elimination patterns. The estimated 95/95 tolerance intervals for a given dose and timepoint combination were then calculated by the following **Equation 5:**

Equation 5

$C_{TI,95/95} = \left(RSD_t * C_{Est,t} * k\right) + C_{Est,t}$, *where*

$C_{TI,95/95}$ *is the estimated* $95/95$ *Tolerance Interval,*

RSD_t = Relative Standard Deviation at time t,

$C_{Est,t}$*is the estimated mean concentration at time* t*, and*

k is the k-factor (for 6 horses, k=3.711)

This equation was used to calculate concentrations of total 3-hydroxymepivaciane at doses of 35 mg, 50 mg, 100 mg, 200 mg, and 400 mg through 168 hours (7 days). Using this equation, we calculated the time post-administration at which urinary concentrations of total 3-hydroxymepivaciane drop below the 10 ng/mL threshold.

RESULTS

The validation of the method employed for the analysis of 3-hydroxymepivacaine contained a calibration curve performed encompassing 50, 500 and 1000 ng/mL 3-hydroxymepivacaine. The response was linear and gave correlation coefficients (R2) of 0.99 or better and glucuronidase hydrolysis was at least 95 % complete. Quality control sample replicates were performed (n = 7). The inter-day accuracy was 10.7% for 50 ng/mL 3-hydroxymepivacaine. The intra-day accuracy was 3.3% for 50 ng/mL 3-hydroxymepivacaine and the precision was 4.9%. The limit of detection (LOD) was 5 ng/mL, and the limit of quantitation (LOQ) was 20 ng/mL, and the method was validated with regard to ion suppression and enhancement.

The urinary data for all six horses are presented in Figure 2, including the mean concentrations of total urinary 3-hydroxymepivacaine at each timepoint and estimated terminal half-lives for total 3-hydroxymepivacaine in urine. The measured total urinary 3-hydroxymepivacaine concentrations were highest at 24 hours post-administration, declining rapidly between 24 and 48 hours, followed thereafter by a slower rate of decline.

The apparent half-life was 7.2 hours between 24 and 48 hours followed post 48 hours by the terminal elimination phase with a much slower 38-hour apparent terminal half-life.

Figure 2.2.1.2 Total urinary 3-Hydroxymepivacaine, 400mg Dose

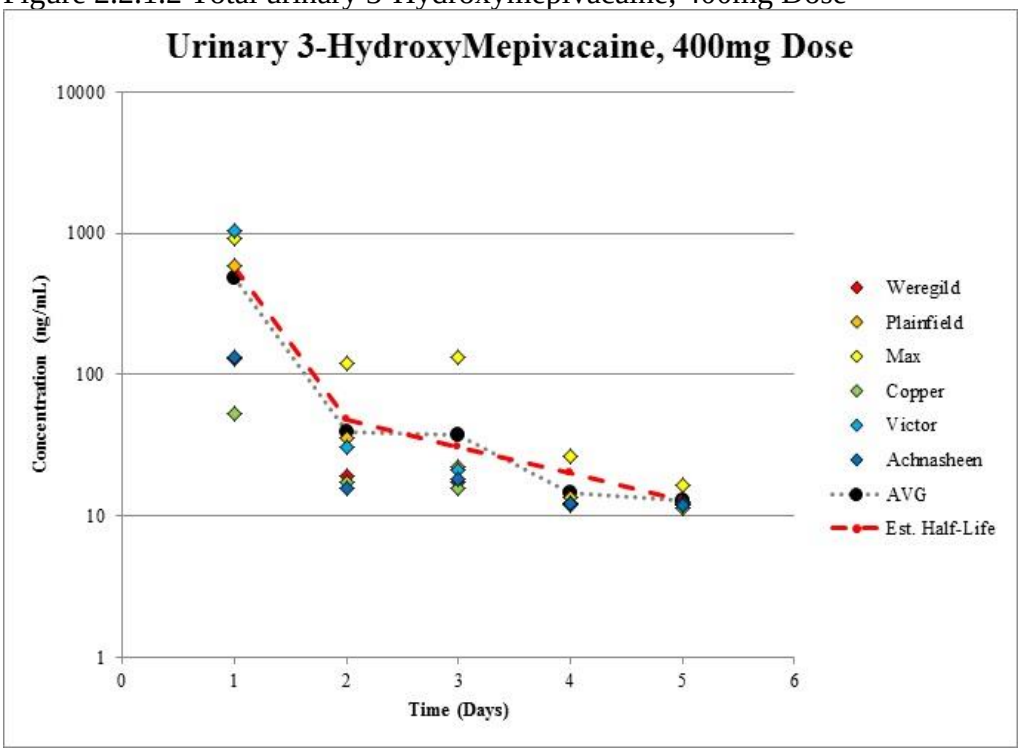

Figure 2: Total Urinary 3-Hydroxymepivacaine, 400 mg Dose: The data points show the resulting total urinary concentrations of total 3-hydroxymepivacaine over 5 days after a 400 mg subcutaneous dose in each individual horse. The maximum total urinary 3-hydroxymepivacaine concentrations were observed at 24 hours post-administration, with a mean urinary concentration of 476 ng/mL. Total 3-hydroxymepivacaine concentration then fell more than ten-fold, with an apparent half-life of about 7.2 hours to yield a mean urinary concentration of total 3-hydroxymepivacaine of about 39 ng/mL at 48 hours post dosing. Post 48 hours the rate of decline of the urinary concentrations of total 3-hydroxymepivacaine slowed considerably, with an apparent terminal half-life of about 38 hours.

Combining these data with those reported from Harkins, et al. 1999 Figure 3 shows that the 400 mg dose data are in good agreement with the earlier study with respect to urinary concentrations of total 3-hydroxymepivacaine in the first 24 hours post-administration.

Figure 2.2.1.3 Total 3-Hydroxymepivacaine, Urine

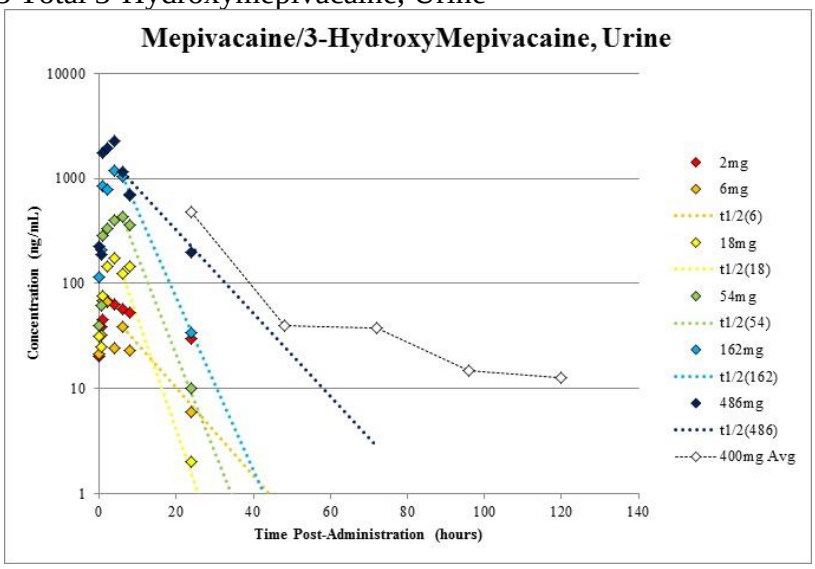

Figure 3: Total 3-Hydroxymepivacaine, Urine: *Showing the combined average data from the 400 mg study with the data reported by* Harkins, *et al*. 1999. *The data show good agreement between both studies on the rate of total 3-hydroxymepivacaine elimination up to 48 hours.*

Using the combined data, Equation 6 was determined to provide a satisfactory fit of total post-administration urinary 3-hydroxymepivacaine concentrations.

Equation 6

$\bar{C}_t \approx \frac{X}{400}(3789e^{-1.11218t} + 2928e^{-0.12698t} + 105e^{-0.01811t} - 6822e^{-1.05637t})$, *where* $\bar{C}_t$ *is the estimated mean total urinary concentration of 3-hydroxymepivacaine at time t,*

X is the subcutaneous dose of mepivacaine in mg, and t is the time post-treatment in hours

The Mean Average Percentage Error [MAPE] for this model is calculated at 27.9%

Graphing Equation 6 and comparing it to the known datasets, there is good overall agreement between the available data sets, as shown in Figure 4. The Mean Average Percentage Error [MAPE] being 27.9% for the overall model.

Figure 2.2.1.4 Total Urinary 3-Hydroxymepivacaine, Model vs Actual

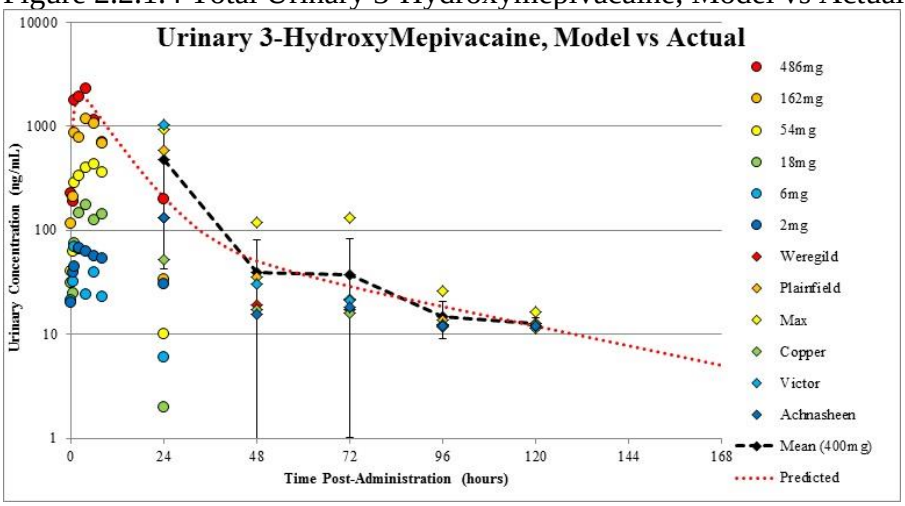

Figure 4: Total Urinary 3-Hydroxymepivacaine, Model vs Actual *shows the fit of the 3-compartment model with production of 3-hydroxymepivacaine via metabolism of parent mepivacaine that was determined by* ***Equation 1*** *for a 400 mg dose. It shows good predictive capabilities. However, adjusting the dose has similar fits that match well with the data from* Harkins, *et al*. 1999.

Table 2.2.1.1 Predicted vs Total Mean 3-Hydroxymepivacaine

Day	Mean	Predicted	Difference	%Difference	Z-Score
0	0	0	0	0	0
1	476.383	206.998	-206.9985	-56.6	-0.62125
2	39.53	50.6213	11.09137	28.1	0.275629
3	37.5733	28.8172	-8.75608	-23.3	-0.19087
4	14.6866	18.4710	3.78441	25.8	0.659708
5	12.695	11.9510	-0.74395	-5.9	-0.41263

Table 1: Predicted vs Total Mean 3-Hydroxymepivacaine: *This table shows the predicted values and compares them to the mean total urinary 3-hydroxymepivacaine values. At all timepoints measured, the model predictions are within 1 standard deviation of the mean.*

These data are well fit by Equation 6, as shown by the data presented in Table # 1, where we compare the concentrations predicted from the mathematical model and the actual concentrations. By day 5 post-administration, the difference between the predicted and the actual values had decreased to -5.9 %, and we also note that total 3-hydroxymepivacaine concentrations were all higher than the 10 ng/mL ARCI threshold. Combining these data into a three-compartment model, Figure 5 presents a general interpretation of the pharmacokinetics of mepivacaine after subcutaneous administration.

Figure 2.2.1.5 General Pharmacokinetic Model for Mepivacaine in Thoroughbreds

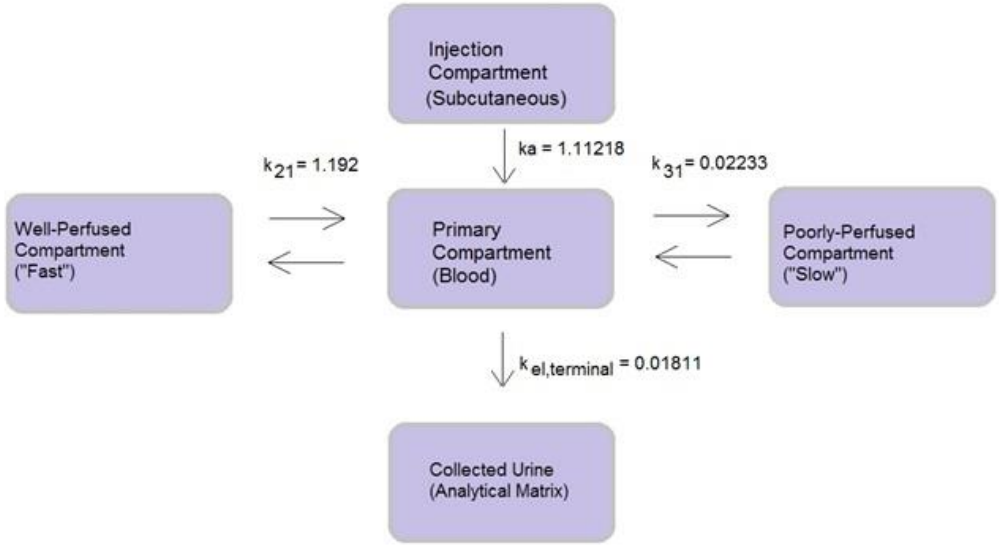

Figure 5: General Pharmacokinetic Model for Mepivacaine in Thoroughbreds *presents the model consistent with the available urinary data for 3-hydroxymepivacaine. Note that the "fast" compartment is considered likely to represent well-perfused tissues such as the liver and central nervous system, while the "slow" compartment is likely to represent less well perfused but high equivalent volume tissues, most likely adipose tissues. This large apparent volume of distribution in the "slow" elimination compartment is presumably*

responsible for the long apparent terminal half-life of urinary total 3-hydroxymepivacaine post subcutaneous administration.

The Relative Standard Deviation (RSD) of total urinary 3-hydroxymepivacaine concentrations also seems to diminish with time in a predictable manner. Graphing this relationship, we see in Figure 6 that the pharmacokinetic parameters from one horse (Max) showed significant individual variance, and so the data are presented both including and excluding the data points from this horse. Clinically, the unusual Max data are most likely the result of an atypical subcutaneous injection site, as absorption following subcutaneous injections can express varying pharmacokinetic behaviors depending on the injection site location (Machin et al, 2019).

Figure 2.2.1.6 Relative Standard Deviation vs Time

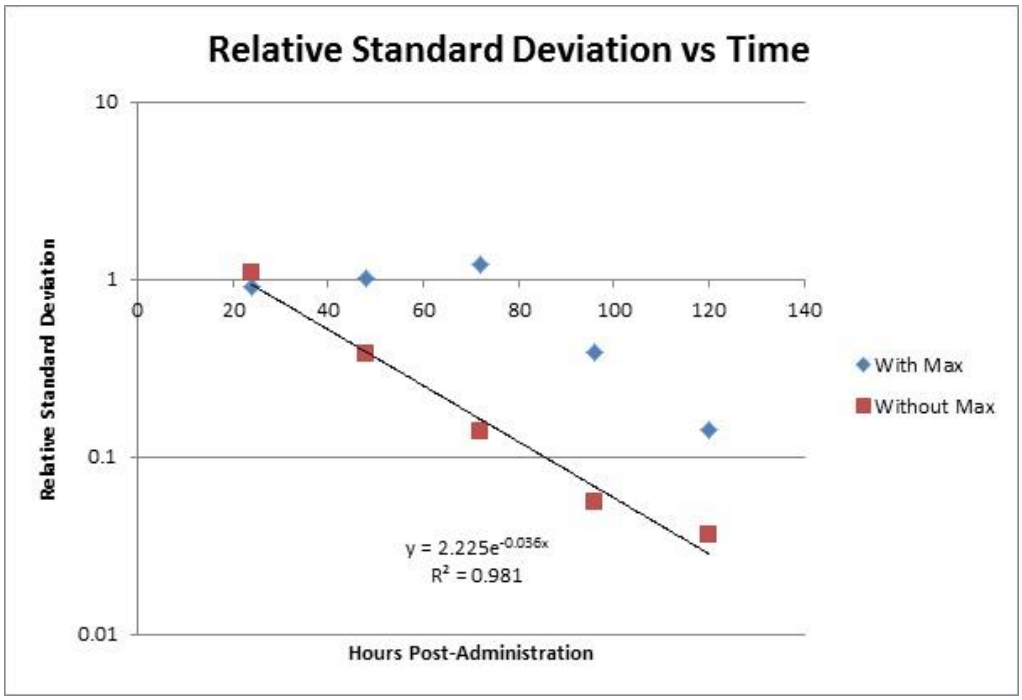

Figure 6: Relative Standard Deviation vs Time *shows the relationship between the standard deviation of the 3-hydroxymepivacaine concentrations in the graph relative to the mean as a function of time. One horse, Max, was characterized by a large amount of individual variance, and so the RSD was calculated with and without data from this horse. In the data excluding him, a strong trend can be seen showing a narrowing of the relative standard deviations between individual urinary concentrations of total 3-hydroxymepivacaine over time. This trend resembles the dataset with Max after the first 72 hours.*

Calculating a 95/95 Tolerance Interval requires knowledge of the mean expected concentration of the analyte and its variance. Combining **Equation 1** with the trend calculated from **Figure 6** for the RSD, a 95/95 Tolerance Interval and a related withdrawal time can be calculated. This was done by multiplying the mean concentrations predicted by **Equation 1** by the RSD at the same timepoint predicted by **Figure 6** to yield an estimate of the Standard Deviation at that timepoint. Doing so for 35 mg, 50 mg, 100 mg, 200 mg, and 400 mg doses produced the following withdrawal time guidelines for practicing veterinarians, as presented in **Figure 7**.

Figure 2.2.1.7 Estimated 95/95 Tolerance Intervals for Various Doses

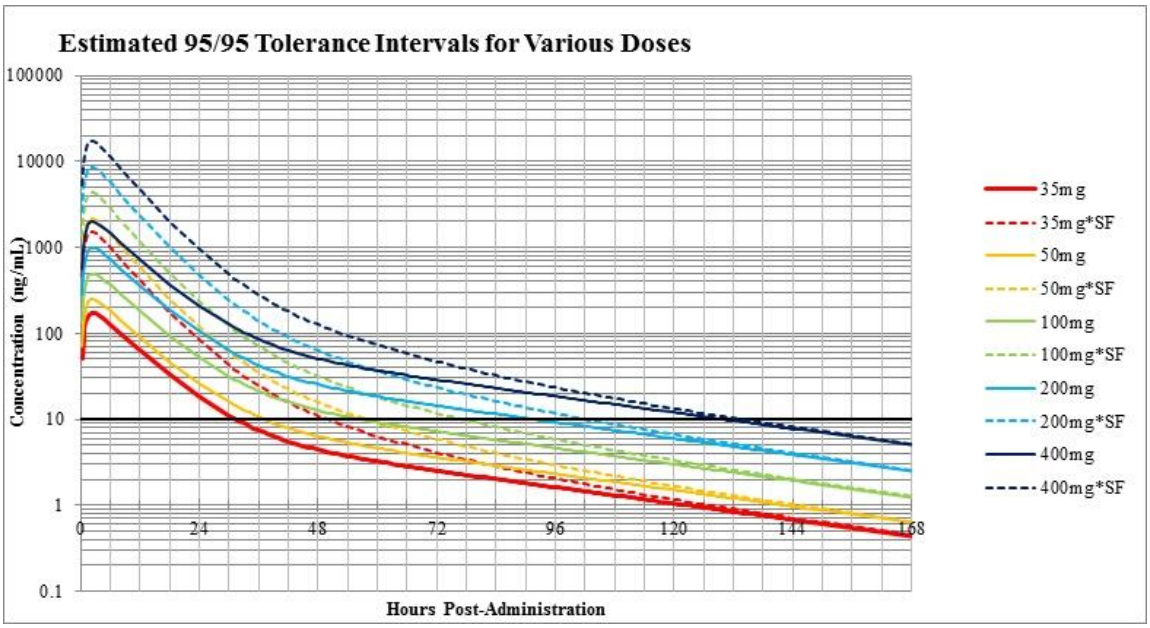

Figure 7: Estimated 95/95 Tolerance Intervals *shows both the predicted mean concentrations (solid lines) as well as the predicted times to reach a 95/95 Tolerance Interval of 10 ng/mL for various doses over a 7-day (168 hour) time course, post-administration. Mean concentrations are denoted by solid lines, while predicted Tolerance Intervals associated with these doses are color-matched in dotted lines, denoted in the legend as "Dose*SF." Each dosing regimen is for subcutaneous administration.*

Of importance for veterinarians is the estimated time at which the urinary concentrations following the doses presented in **Figure 7** would decline to less than the 10 ng/mL regulatory threshold. This information is presented below in **Table 2**. All times are reported as the minimum time (in hours) for a given dose to reach a mean total urinary 3-hydroxymepivacaine concentration of 10 ng/mL or until a time at which a 95/95 Tolerance Interval would yield 10 ng/mL.

Table 2.2.1.2 Withdrawal Guidelines for 95/95 Tolerance Interval for a Regulatory Threshold of 10 ng/mL

Dose (mg)	Time (Hours) to Mean Conc. 10 ng/mL	Upper Limit of the 95/95 Tolerance Interval for the Time (Hours) to 10 ng/mL
35	32	50
50	38	58
100	57	77
200	92	103
400	130	134

Table 2: **Withdrawal Guidelines for a 95/95 Tolerance Interval for a regulatory threshold of 10 ng/mL** *shows the predicted time for various doses to reach a mean concentration 10 ng/mL of total 3-hydroxymepivacaine in urine and the time associated for that dose in a population to produce a Tolerance Interval of 10 ng/mL for 3-hydroxymepivacaine in urine. Veterinarians are advised to use the right-hand column as a general guideline when estimating withdrawal times.*

DISCUSSION

The goal of this study was to develop best estimates of the urinary concentrations of total 3-hydroxymepivacaine that would be expected following administration of clinically relevant doses of mepivacaine larger than the ARCI guideline dose of 0.07 mg/kg., about 35 mg/horse. Jurisdictions outside the United States recognize that clinically relevant doses may at times exceed the ARCI 35 mg dose Regulatory guidelines for mepivacaine administration in other jurisdictions include a 48 hour withdrawal for a SQ dose of 300 mg in Canada (Agriculture Canada, 2016), a 48 hour detection time for 400 mg in Australia (AEVA,1992), and a 4.5 day detection time for SQ dose of 0.395 mg/kg,

about 180 mg, by the European Horseracing Scientific Liaison Committee (EHSLC) (European Horserace Scientific Liaison Committee, 1997). These guidelines were developed without a reference standard for 3-hydroxymepivacaine, which was first synthesized as a research standard by the Kentucky Equine Medication Research Program, which research led to an invited review in the *Journal of Veterinary Pharmacology and Experimental Therapeutics* which review supported the use of quantitative urinary thresholds as scientifically and forensically appropriate (Tobin *et al*, 1999).

The results reported here are in good agreement with published regulatory and related research on mepivacaine as well as with current regulatory practice. Tobin *et al* (1999) reported that dosing with the defined Highest No Effect Dose (HNED) of 2 mg of mepivacaine yielded a peak urinary concentration of total 3-hydroxymepivacaine of about 65 ng/mL. Similarly, review of the ELISA data obtained following administration of a sequence of subcutaneous mepivacaine doses ranging from 2 to 483 mg showed that the bulk of an administered dose of mepivacaine is rapidly eliminated in the first hours post administration (Harkins, *et al* 1999). Consistent with this pattern, the urinary concentrations of total 3-hydroxypmepivacaine determined following administration of the 400 mg dose in this study were all above 10 ng/mL at 72 hours post-dosing and also remained above this 10 ng/ml threshold at 120 hours post dosing.

CONCLUSION

The urinary elimination of total 3-hydroxymepivacaine following subcutaneous injection of a 400 mg dose follows a multi-compartmental model with mepivacaine rapidly diffusing from the subcutaneous injection site into the central plasma compartment and then distributing widely throughout the horse. Mepivacaine then sequesters into other high-volume compartments, most likely adipose tissue, because of the lipophilicity of the drug – mepivacaine has a pKa of 7.6 (Becker & Reed 2006), when compared to the pH of 7.4 of equine blood, this indicates a ratio of the charged-to-uncharged of approximately 0.63. That is, approximately 60% of the mepivacaine in blood will be found in the lipid-soluble form. The data presented in **Table 2** and **Figure 7** provide the to date best available guidance to veterinarians using higher doses of mepivacaine than the 35 mg dose in the

ARCI guidelines. Based on the data reported herein and rounding out to at least the next 24-hour time point, it would be unwise to suggest withdrawal times of less than 96 hours for a 100 mg dose, 120 hours for a 200 mg dose, and, allowing an extra 24 hours at this higher dose, 168 hours for a 400 mg dose.

ACKNOWLEDGEMENTS

This research is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch Program under project KY014051 Accession Number 1010609 and also by grants from the USDA Agriculture Research Service Specific Cooperative Agreement #58-6401-2-0025 for Forage-Animal Production Research, the Kentucky Department of Agriculture and the Kentucky Thoroughbred Association Foundation and by support for the Kentucky Agricultural Experiment Station as provided by the National Institute of Food and Agriculture (NIFA) and the Commonwealth of Kentucky. Other support that has made this work possible includes research support from The Equine Health and Welfare Alliance, Inc, Versailles, Kentucky, the National Horsemen's Benevolent and Protective Association and the Alabama; Arizona; Arkansas; Ontario, Canada; Charles Town, WV; Florida; Indiana, Iowa; Kentucky; Louisiana; Michigan; Minnesota, Nebraska, Ohio, Oklahoma, Ontario Canada, Oregon, Pennsylvania, Tampa Bay Downs, Florida; Texas; Washington State; and West Virginia Horsemen's Benevolent and Protective Associations and Mrs. John Hay Whitney. Published as paper #492 from T Tobin and the Equine Pharmacology, Therapeutics and Toxicology Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky.

CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

REFERENCES

Agriculture Canada (1991) Racetrack Division Schedule of Drugs. Minister of Agriculture, pp. 5 83. Agriculture Canada, Ottawa, Ontario.

Australian Equine Veterinary Association (1992) Detection of Substances in Racing Horses, pp. 8 49. Australian Equine Veterinary Association, Artarmon, Australia.

Becker, D. E., & Reed, K. L. (2006). Essentials of local anesthetic pharmacology. Anesthesia progress, 53(3), 98-109.

California, S. O. (n.d.). Query Rules & Regulations Results. Retrieved March 01, 2018, from http://www.chrb.ca.gov/query_rules_and_regulations_database.asp?form_query_action=display_rule&form_query_rule_number=1844&form_query_rule_title=Authorized%2BMedication.&form_query_article=Authorized%2BMedication.&form_query_article_index=17&form_query_argument=1844

European Horserace Scientific Liaison Committee (1997) Information for Veterinary Surgeons on Detection Periods of Named Drugs, R & W Publications Limited, Newmarket, UK.

Harkins, J. D., Karpiesiuk, W., Woods, W. E., Lehner, A., Mundy, G. D., Rees, W. A., ... & Tobin, T. (1999). Mepivacaine: its pharmacological effects and their relationship to analytical findings in the horse. *Journal of veterinary pharmacology and therapeutics*, 22, 107-121.

KHRC. (2015). Kentucky Horse Racing Commission Withdrawal Guidelines Thoroughbred; Standardbred; Quarter Horse, Appaloosa, and Arabian KHRC 40-02. http://khrc.ky.gov/Documents/KHRC%2040-02-Withdrawal%20Guidelines.pdf (accessed Nov 27, 2016).

Machin, J., Duer, W., Maylin, G., Fenger, C., Wilson, D., Ivey, M., ... & Tobin, T. (2019). Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: A field study. Equine veterinary journal, 51(3), 343-348.

Tobin, T. (1981). Drugs and the performance horse. Charles C. Thomas.

Tobin, T., Dirikolu, L., Brewer, K., & Hughes, C. G. (2013). A clinician's guide to factors affecting withdrawal times for equine therapeutic medications. *The Veterinary Journal*, 198(2), 313-321.

Tobin, T., Harkins, J. D., & Sams, R. A. (1999). Testing for therapeutic medications: analytical/pharmacological relationships and 'limitations' on the sensitivity of testing for certain agents. *Journal of veterinary pharmacology and therapeutics*, 22(3), 220-233.

Toutain P. L. (2010). How to extrapolate a withdrawal time from an EHSLC published detection time: a Monte Carlo simulation appraisal. *Equine veterinary journal*, 42(3), 248-254.

Woods, W. E., Lehner, A. F., Karpiesiuk, W., Boyles, J., Carter, W., Harkins, J. D., & Tobin, T. (1998). Detection and Quantitation of 3-Hydroxymepivacaine in Horse Urine: A Preliminary Report. Proceedings of the 12th International Conference of Racing Analysts and Veterinarians, 324-328.

2.2.2 Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: A field study

Abstract: Background: Methylprednisolone (MP) acetate is a commonly used corticosteroid for suppression of inflammation in synovial structures in horses. Its use is often regulated in equine sports by plasma MP concentrations. Objectives: This study seeks to identify variables that may influence MP clearance when used therapeutically in equine athletes in regulated sports. Racing discipline, co-administration with hyaluronic acid (HA) and synovial structure were investigated. Study Design: Field study in actively racing horses in three disciplines (Thoroughbred, Standardbred and Quarter Horse). Methods: Seventy-six horses (15 Thoroughbreds, 20 Standardbreds, 41 Quarter Horses) were included in the study. Injection of any synovial structure with a total body dose of 100 mg MP acetate was permitted. Coadministration with Hyaluronic acid (HA) was recorded. Plasma was collected before injection and at 6 days post-injection within two hours of the injection time and analyzed by Liquid Chromatographic tandem Mass Spectroscopy. Percent censored data for each synovial structure was determined, and summary statistics generated by Robust Regression on Order. Differences between synovial structures and co-administration with HA were identified by t-test. Results: The plasma concentration for the entire group (mean ± standard deviation (sd), pg/mL) was 96 ± 104. The high motion (HM) group had a lower plasma MP concentration.

Experimental: As above, seventy-six horses (15 TB, 20 SB, 41 QH) were included in the study. Injection of any synovial structure with a total dose of 100mg MP acetate was permitted. Co-administration with HA was recorded. Plasma was collected before administration and at 6 days post-injection within two hours of the initial injection time. Analysis was via LC-MS/MS. Robust Regression on Order statistics were generated for summary data and censored percentages were included for each injection site.

Conclusion: Injection into the Distal Tarsal Subchondral, Chondroitin Carpal Sheath, and Medial femorotibial joints as well as combination therapy with Hyaluronic acid may

prolong apparent clearance and so longer withdrawal guidelines should be observed when appropriate.

Citation: Machin J, Duer W, Maylin G, Fenger C, Wilson D, Ivey M, Berthold B, Allison S, Tobin T. Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: A field study. Equine Vet J. 2019 May;51(3):343-348. doi: 10.1111/evj.13003. Epub 2018 Sep 5. PMID: 30080939.

Personal Contribution: Data analysis of non-censored data points and between censored/non-censored groups, statistical work (Gauss-Camp-Meidell done by associate while training me on theory), collaborated with veterinarian in design of initial data collection, large contribution to writing of manuscript

Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: A field study

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Summary

Background: Methylprednisolone (MP) acetate is a commonly used corticosteroid for suppression of inflammation in synovial structures in horses. Its use is often regulated in equine sports by plasma MP concentrations.

Objectives: To describe variability in MP plasma concentrations after MP acetate injection in different synovial structures and with co-administration with hyaluronic acid (HA).

Study design: Field study in actively racing horses in three disciplines (Thoroughbred, Standardbred and Quarter Horse).

Methods: Seventy-six horses (15 Thoroughbreds, 20 Standardbreds and 41 Quarter Horses) were included in the study. Injection of any synovial structure with a total body dose of 100 mg MP acetate was permitted, data were grouped according to the synovial structure injected and co- administration with HA. Plasma was collected before injection and at 6 days post-injection. Per cent censored data (below the limit of quantification) for each synovial structure were determined, and summary statistics generated by Robust Regression on Order. Differences between synovial structures and co-administration with HA were identified by ANOVA with Tukey's post hoc testing.

Results: The MP plasma concentration at 6 days for injection for the entire group (mean standard deviation [s.d.], pg/mL) was 96 104. Metacarpophalangeal (MCP) plasma concentrations contained 86% censored data and could not be included in the statistical analysis. The carpal joints (CJO) group had a lower plasma MP concentration ($P<0.05$) than the distal tarsal joints (DTJ) or medial femorotibial (MFT), the no HA (NHA) group had a lower plasma MP concentration ($P<0.05$) than HA.
Main limitations: The synovial structures injected varied by racing discipline, so this study was unable to identify any differences between disciplines. Conclusions: Practitioners should be aware that injection of DTJ, CS and MFT joints, and combining MP acetate with HA may prolong its clearance, and withdrawal times for competition in regulated equine sports.

Keywords: horse; methylprednisolone; joint injection; racehorse; threshold; plasma concentration

Introduction

Methylprednisolone acetate, pregna-1,4-diene-3,20-dione, 21-(acetyloxy)-11,17-dihydroxy-6-methyl-(6a,11b), molecular weight 416.51 g/mol (MP acetate) (Depo-Medrol®) a is an FDA-approved long-acting, slightly water- soluble corticosteroid prodrug for intrasynovial administration in horses where targeted therapy is recommended. The marginal water solubility of MP acetate delays its entry into joint fluid, accounting for its prolonged therapeutic action. Once dissolved in joint fluid the acetate prodrug moiety is catalyzed by alkaline hydrolysis/esterase action, yielding the therapeutically active molecule, methylprednisolone.

Over 53% of racehorses [1] experience lameness during their racing careers, of which joint injury is one of the major causes. As many as 27% of Thoroughbred yearlings go through public auction with pre-existing arthritis [2], and this problem is not limited to Thoroughbreds, as a comparable 33% prevalence is seen in nonracing breeds [3]. Targeted joint therapy for these conditions using MP acetate has been a mainstay of equine veterinary care since the early 1960s. Use of this specific therapeutic medication is accompanied by restrictions in proximity to competition in performance horses with most international sports and racing jurisdictions providing withdrawal guidelines without

published thresholds. The Canadian Para-Mutuel Agency (CPMA) recommends a 6- day withdrawal after 100-mg intra-articular (IA), or a 14-day withdrawal after 200 mg [4], whereas the Federation Equestre Internationale provides a detection time of 14 days after 100-mg IA and 28 days after 200-mg IA [5].

In the United States, many jurisdictions have adopted a regulatory threshold of 100 pg/mL MP in plasma or serum pursuant to a recommendation by the Racing Medication and Testing Consortium (RMTC), which is accompanied by a withdrawal recommendation of 21 days. The RMTC typically determines thresholds based on the application of a statistical method called the 95/95 tolerance [6], although it is not clear whether this statistical method was used in the case of MP. The RMTC references a pharmacokinetic study of 16 research horses [7], in which MP acetate injection was restricted to a single dose of 100 mg without concomitant medication in one antebrachiocarpal joint of Thoroughbreds, and these data were used to determine both MP plasma pharmacokinetics and support their threshold recommendation. The regulated population of racing horses that receive MP acetate may have injections into joints other than the antebrachiocarpal joint and is comprised of horses other than Thoroughbreds. In the case of at least one therapeutic medication, glycopyrrolate, pharmacokinetic properties differ between Thoroughbreds and Standardbreds [8].

In order to provide guidance for horsemen and veterinarians operating under regulatory restrictions, including the effect of synovial structure being injected and co-administration of hyaluronic acid, this study was performed. We sought to include horses of all three major racing disciplines, Thoroughbreds, Standardbreds and Quarter Horses under actual training conditions and to evaluate the effect of the widely used co-administration with hyaluronic acid (HA) in order to provide guidance to practitioners for practical therapeutic use of MP acetate. We chose 6 days post-MP acetate administration for collection of plasma samples because this time frame was likely to minimize the censored data points, or data below the limit of quantification (LOQ), based on the previous work [7].

Materials and methods

Study facilities and animals

Privately owned Thoroughbred, Standardbred and Quarter Horse racehorses in race training in the practice population of five of the authors (C.F., D.W., M.I., B.B. and S.A.) were used throughout. Horses were stabled on the racetrack or at training centers and were housed and trained according to standard procedures at racing facilities in Kentucky, Ohio and New Mexico. The feed, bedding and water sources were consistent with routine management at each facility. Training adhered to regimens consistent with the type of racing specific to the racing discipline. Informed consent was obtained for all horses enrolled. Inclusion criteria were a full clinical examination and a diagnosis requiring joint and/or synovial compartment therapy with 100-mg MP acetate total body dose. Treatment was based on the exam and diagnosis by the examining investigator, a signed owner consent form, long-term trusted relationships between the investigator and trainer to ensure trainer compliance and active participation in racing or fast workouts in preparation for racing. Exclusion criteria were previous injections with MP acetate within 1 month, or any other medications within the 24 h prior to blood collection.

Experimental design

All racehorses in the five investigators' practices which fulfilled the inclusion criteria with none of the exclusion criteria were enrolled in the study. In order to replicate the usual clinical usage patterns of MP acetate, no restrictions were placed on co-administered medications. The synovial structure injected was recorded, without any restrictions on which structures could be injected. The injected structures were the medial femorotibial joint (MFT), distal intertarsal joint and tarsometatarsal joint (DTJ), distal interphalangeal (DIP), antebrachiocarpal and intercarpal (combined as CJO), metacarpophalangeal joints (MCP) and carpal sheath (CS). Blood was collected into lithium heparin tubes prior to synovial structure injection(s), and post-injection blood was collected in all cases 6 days (2 h) after synovial structure injection. In five cases where preinjection samples were not collected, a complete review of the horse's medical record for the last month was performed to ensure that no prior injection with MP acetate had occurred. Blood was kept refrigerated at 4°C and shipped overnight to the New York Drug Testing and Research Laboratory for analysis.

Analytical methods

The analytical procedure followed was the ISO 17025/RMTC accredited quantitative analytical procedure for Methylprednisolone in place in the New York Drug Testing and Research Program. The reference standard for MP was purchased from Sigma Aldrich. The analytical reference standard MP-d2 used as internal standard was purchased from CDN Isotopes. Stock solutions of MP and the internal standard were prepared at 1 mg/mL in methanol. Acetonitrile and methanol were purchased from EMD Millipore, and methyl-tert-butyl ether and ammonium formate were purchased from Fisher Scientific. Deionized water was filtered onsite to the specification of 18.2O. All reagents were HPLC grade or better.

Methylprednisolone working solution was prepared by dilution of the 1 mg/mL stock solution with ethanol to the concentration of 25 pg/mL. Plasma calibrators were prepared by dilution of the working standard solution with drug-free plasma to concentrations of 50, 100 and 200 pg/ mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay.

Prior to analysis, 1 mL of plasma was aliquoted into new, labelled test tubes. To each tube, MP-d2 stock solution was added along with 5 mL of methyl-tert-butyl ether. The samples were mixed by rotation for 10 min, centrifuged at 2400 rpm for 5 min and the top ether layer removed and dried under nitrogen. Samples were dissolved in 100 mL of 1:1:1 acetonitrile: methanol: DI water and a 2.5-lL aliquot injected into the LC-MS/ MS system, Agilent 6400 series triple quadrupole mass spectrometer coupled with an HPLC chromatography system.

The concentration of MP was measured in plasma by LC-MS/MS using positive electrospray ionization. The mass spectrometer was operated using electrospray combined with Agilent's Jet Stream Technology. Chromatography employed a Zorbax SB-C18 column with specifications of 10 cm 9 3.0 mm, 3.5 mm, column and a linear gradient of acetonitrile (ACN) in water with a constant 5 mmol/L ammonium formate (pH 3.5) at a flow rate of 0.5 mL/min. The initial ACN concentration was held at 40% for

3.0 min, ramped to 95% over 1.0 min and held at that concentration for

1.0 min before re-equilibrating for 0.5 min at initial concentration.

Detection and quantification were conducted using selective reaction monitoring (SRM) of initial precursor ion for MP (mass-to-charge ratio

375.2 m/z) and the internal standard (361.2 m/z). The response for the product ions for MP (m/z 339, 321, 293 and 253) and the internal standard (m/z 161) was plotted and peaks at the proper retention time integrated using MassHunter software. MassHunter software was used to generate calibration curves and quantitate MP in all samples by linear regression analysis.

The validation of the method employed for the analysis of MP contained a calibration curve performed encompassing 50, 100 and 200% of the threshold value for MP. The response was linear and gave correlation coefficients (R2) of 0.99 or better. Quality control samples replicates were performed (n = 7). The interday accuracy was 5.5% for 100 pg/mL MP. The intraday accuracy was 0.3% for 100 pg/mL MP. The interday precision was 2.5% for 100 pg/mL MP. The intraday precision was 13.9% for 100 pg/mL MP. The technique was optimized to provide a lower limit of quantification (LOQ) of 0.05 ng/mL. The limit of detection (LOD) was 0.025 ng/mL. This analytical method has been shown to exclude 20-dihydro-6-methylprednisolone, an isomeric metabolite of methylprednisolone in the horse and a possible confounding isomeric metabolite of methylprednisolone with respect to the unequivocal identification and confirmation of methylprednisolone in post- MP administration equine plasma samples [9].

Data analysis

The 6-day post-administration plasma MP concentrations were analyzed for percentage of censored (below LOQ) data, effects of number of synovial structures injected, specific synovial structure injected and coadministration with HA using Robust Regression on Order [10] and General Linear Model statistical methods [11]. Data were first grouped as all horses, then subgrouped according to the synovial structure, number of synovial structures injected and concomitant administration of HA. Where multiple injections resulted in horses being categorized into more than one group, the data from the overlapping groups were compared with each individual group in order to determine if these data should be compared separately. The effect of HA on plasma clearance was also

included as a separate analysis of co-administration with HA (HA) or no co-administration with HA (NHA).

Each dataset was first analyzed for percent censored data, then Normality tests (Shapiro-Wilk, Anderson-Darling, Lillefors and Jarque-Bera) were performed on uncensored (above LOQ) data in order to determine the most appropriate statistical analysis for threshold determination. Summary statistics for each data subgroup were obtained for all groups except CS data and MCP data using RROS [11] in R-programming language and bootstrap analyses with 100,000 resamplings (XLSTAT®, ADDinsoft 2016 https://www.xlstat.com/en/ as an Excel® for Mac 2011, Microsoft add- in). Bootstrap with resampling was performed to improve estimates of group summary statistics. Comparisons of the grouped Synovial structure data were then performed using ANOVA, with Tukey's post hoc testing.

The use of concomitant HA and no HA groups was compared in a separate analysis using a t test. The effect of the number of joints injected was compared using joints as count data with a General Linear Model in R being cognizant of the effects of any overdispersion [11], and homoscedasticity of variances tested by Bartlett and Brown-Forsythe tests [12]. Thresholds for each data set were determined using two different methods: (95/95) tolerance interval [6] and Gauss-Camp-Meidell (GCM) [13].

Results

Seventy-six horses met the inclusion criteria (15 Thoroughbreds, 20 Standardbreds and 41 Quarter Horses). Sixty-eight horses had preinjection plasma samples analyzed, and all of these plasma samples were below the LOD of the analytical method for MP. Seven horses did not have pre- injection plasma samples analyzed, but a review of the medical history for these horses showed no previous MP administration or exposure, and these horses were included in the analysis.

The MCP data set had 86% censored data, so no further analysis could be conducted [10]. In all cases where the DIP joint was injected, the MCP joint was also injected, so no separate conclusions or analyses could be done on this subgroup. There were 13 instances of horses categorized into both the MCP and CJO groups. Of these, 12 were censored.

There was one instance of a horse categorized into the MFT and CJO group (censored), and one instance of a horse categorized into the MFT and DTJ groups (uncensored). All 15 were included in both groups for analysis. The per cent censored data for each group is shown in Table 1. Normality tests (Shapiro-Wilk, Anderson-Darling, Lillefors and Jarque-Bera) for each data set (All horses, CJO, CS, DTJ, MFT, HA and NHA) indicated that non-censored data (plasma concentrations above the LOQ) were normally distributed.

Where censored data in groups fell below 80% (All horses, CJO, CS, DTJ, MFT, HA and NHA groups), summary statistics for plasma concentrations arising from MP acetate intrasynovial injections were obtained as described above. Standard parametric summary statistics were used for CS data, as recommended by Helsel [10]. Box and whisker plots (mean, interquartile range and highest and lowest scores, with outliers indicated) for these data are shown in Figures 1 and 2, and differences indicated in the figures.

Breed/racing discipline differences were found among which specific synovial structures were commonly injected with MP. Insufficient horse numbers were present for each joint or synovial structure injected among the different breeds, and not every breed was represented in all injection groups (Table 2). Therefore, all breeds were combined, and the differences were analyzed by the MP-treated synovial structure. No effect of the number of synovial structures injected on the MP plasma concentrations was found (Figure 3, $P = 0.8$) with lack of overdispersion. There were 13 instances where CJO and MCP were co-injected and one instance where DTJ and MFT were co-injected. In all cases, the resulting MP plasma concentrations were within the interquartile range of both groups, so the case was included in the analysis of both groups.

Table 2.2.2.1 Distribution of censored and Uncensored Data (Methylprednisolone)

Injection site	Total	#Censored	%Censored	%Uncensored
All horses	76	46	61	39
Carpal joints	36	25	69	31
Carpal sheath	4	0	0	100
Distal tarsal joints	7	1	6	94
Metacarpophalangeal	34	29	85	15
Medial femorotibial	10	4	40	60
Hyaluronic acid	21	10	48	52
No hyaluronic acid	55	36	65	35

TABLE 1: The distribution of censored (plasma concentrations methylprednisolone below the limit of quantification) and uncensored (plasma concentrations methylprednisolone at or above the limit of quantification) among 6-day methylprednisolone plasma concentrations. Horses that were injected into multiple synovial structures are included in both groups and include 13 horses in both the metacarpophalangeal and carpal joints, one horse in both the medial femorotibial and carpal joints and one horse in both the medial femorotibial and distal tarsal joints

Figure 2.2.2.1 Box & Whisker plots for Plasma Methylprednisolone by Joint

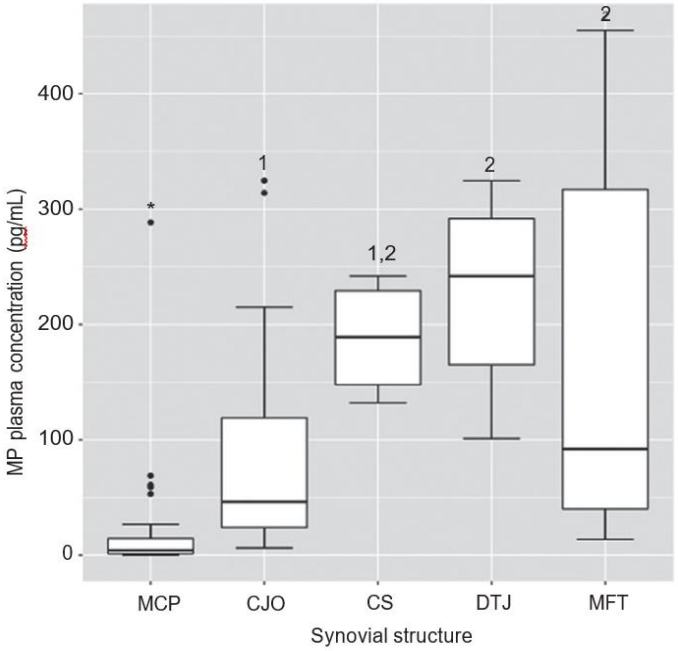

*Figure 1: Box and whisker plots for plasma methylprednisolone concentrations 6 days post-injection by synovial structure. Concentrations that differ by structure are indicated by different letters. *Metacarpophalangeal data are shown for comparison but were not included in the analysis because the censored data (below the limit of quantification) exceeded 80%, precluding this group from analysis [10].*

Figure 2.2.2.2 Box and Whisker Plots for Methylprednisolone by Administration of HA

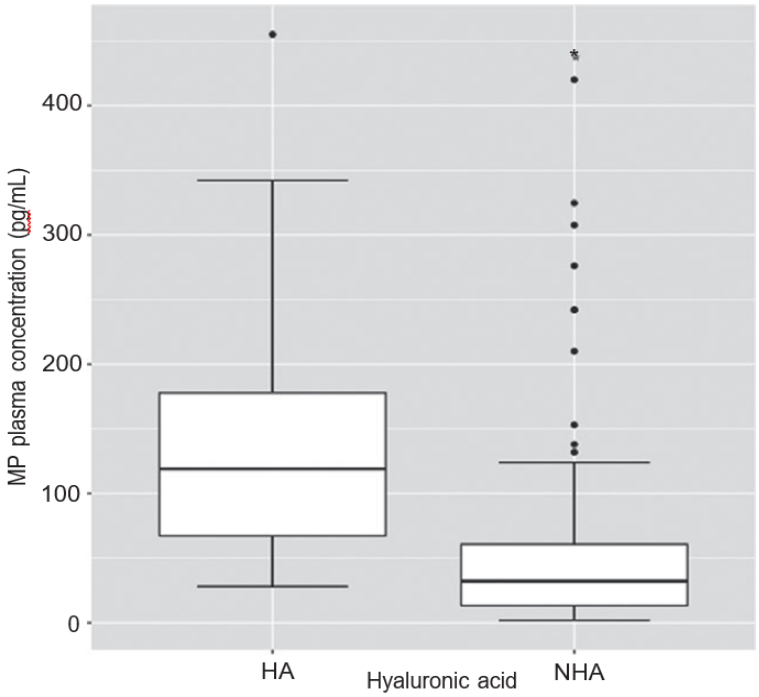

Figure 2: Box and whisker plots for plasma methylprednisolone concentrations 6 days post-injection by concomitant injection of hyaluronic acid compared with no hyaluronic acid. The addition of hyaluronic acid to the injection doubled the methylprednisolone plasma concentration.

The plasma concentration for the entire group (mean ± s.d., pg/mL) was 96 ± 104. The CJO group had a lower plasma MP concentration (53 ± 68, $P<0.05$) than the DTJ (226 ± 74) and the MFT (177 ± 154) group, and no difference was identified among the other groups evaluated. The NHA (75 ± 96) group had approximately half the MP concentration of the HA (147 104, $P<0.05$) group. Six-day tolerance limits or thresholds generated using 95/95 or GCM ($P = 0.05$, $P = 0.01$) are presented in Table 3.

Table 2.2.2.2 Synovial Structures Injected by Breed

Breed	Carpal joints	Carpal sheath	Distal tarsal joints	Metacarpophalangeal joint	Medial femorotibial joint	Hyaluronic acid	No hyaluronic acid
Thoroughbred	4	0	6	4	1	7	8
Standardbred	8	4	0	5	3	14	6
Quarter Horse	24	0	1	20	6	0	41

TABLE 2: Synovial structures injected by breed

Figure 2.2.2.3 Number of Synovial Structures Injected vs Plasma Methylprednisolone

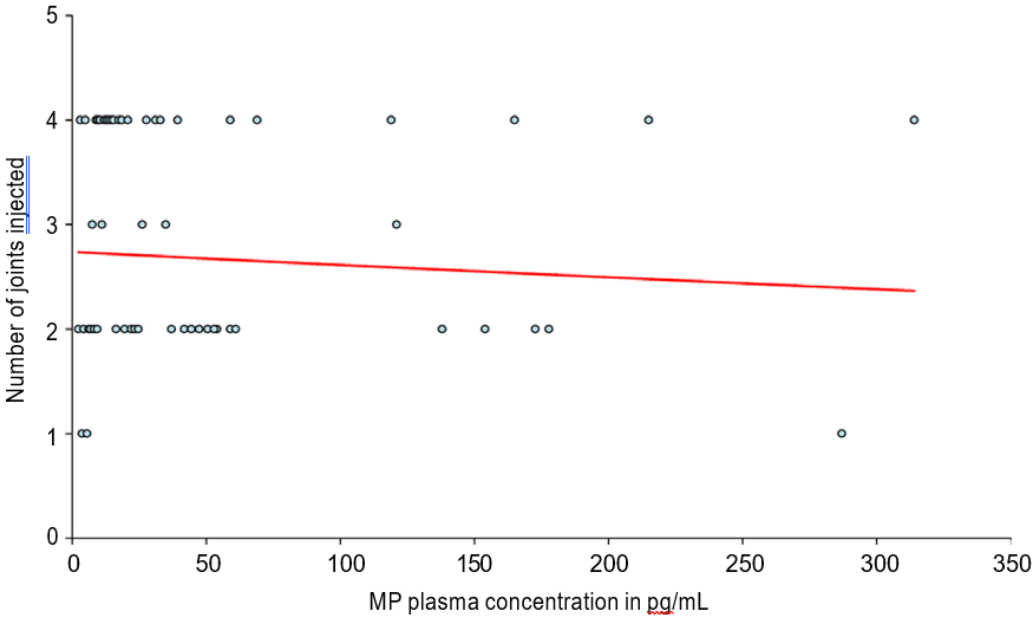

Figure 3: Number of synovial structures injected and plasma methylprednisolone concentrations 6 days post-injection (demonstrates no effect with P = 0.8).

Table 2.2.2.3 Tolerance Limits or Thresholds for 6-day Methylprednisolone

Synovial structure	N	95/95	Gauss-Camp-Meidell (P = 0.05)	Gauss-Camp-Meidell (P = 0.01)
All horses	76	487	407	791
Carpal joints	37	418	416	770
Carpal sheath	4	675	312	455
Distal tarsal joints	7	890	450	721
Metacarpophalangeal joint	34	245	199	398
Medial femorotibial joint	10	3160	636	1202
Hyaluronic acid	21	647	460	844
No hyaluronic acid	55	534	366	719

TABLE 3: Tolerance limits or threshold values (pg/mL) for a 6-day methylprednisolone plasma concentration for two different statistical methods. The Gauss-Camp-Meidell method (P = 0.05) threshold was chosen to match the 95/95 risk of a positive test, and the Gauss-Camp-Meidell method (P = 0.01) is included to demonstrate a threshold for a risk of 1 in 100 tests

Discussion

This population study in racing horses was undertaken to evaluate the post-MP acetate administration plasma concentrations of MP under field conditions. Our findings provide guidance for practitioners when deciding upon MP acetate use in different synovial structures and demonstrate the usefulness of different statistical methods for the determination of thresholds for therapeutic substances. The results concur with and support previous findings [7] that the pharmacokinetics of MP differs depending upon which synovial structures are treated. Knych et al. [7] demonstrated differences in MP plasma pharmacokinetics between the antebrachial (AC) joint and the intercarpal (IC) joints, whereas our study demonstrates differences among CJO, DTJ and MFT. We were unable to repeat the findings of the previous Knych et al. [7] study because the AC and IC joints were commonly injected together in the practices of our investigators. Therefore, any differences between the pharmacokinetics of MP between these two specific joints would not be detected in our study design.

Metacarpophalangeal joints were associated with the most rapid clearance of MP from the plasma in this study (Figure 1), with 85% of the data falling below the LOQ (censored), and only a single horse exceeding the 21- day RMTC recommended threshold of 100 pg/mL. The high percentage of censored data in this group prevented any statistical analysis of this group. The DIP is included in the MCP group because in all cases (N = 5) in our study the two structures were injected together. Of these five horses, all were well below the 100 pg/mL threshold and 60% of the data were censored. It is likely that this joint shares the characteristics of rapid clearance from the plasma with the MCP. This may result from its considerable range of motion of 46–47 degrees of flexion/extension despite being encased in the hoof [14].

Of the joints that could be included in the statistical comparisons, the CJO had the lowest MP plasma concentrations. In the MCP, DIP and CJO joints, the low plasma concentrations of MP at 6 days post-administration may have been associated with the relative ease of the injection procedure resulting in the entire dose being deposited into the joint, the motion of the joint which could be associated with increased blood flow or high rates of mechanical disruption of the actual MP acetate particles. In contrast, DTJ injections were associated with the highest plasma MP concentration at 6 days post-injection. This

may have resulted from a larger proportion of the MP acetate being deposited outside of the joint in the subcutaneous tissues, reflux of the injectate back out through the needle track and into the subcutaneous tissue because of the anatomy of the synovial structure, or simply a lower rate of mechanical disruption of the MP acetate particles. The DTJ are characterized by minimal joint fluid volume, and even small volumes of medication injected into the joint would likely cause sufficiently increased pressure in the joint to result in significant reflux. This likelihood that a portion of MP refluxed back through the needle track is supported by the narrow standard deviation for plasma MP concentrations for DTJ, reflecting the consistency with which this higher plasma concentration is observed. In contrast, the plasma MP concentration after injection of the MFT joint was accompanied by both a high mean and standard deviation, possibly reflecting the technical difficulty of the injection procedure, or variable uptake of the MP into the infrapatellar fat pad. If any portion of the dose of MP is inadvertently delivered outside the joint/synovial cavity, a longer withdrawal can be expected. Slight movement of the horse during the MFT injection procedure could readily result in portions of the dose administered being deposited peri-articularly. The plasma MP concentrations from horses that had CS synovial structures treated had a similar profile to the distribution of MP from those with DTJ injections, suggesting a similar mechanism for medication uptake into the systemic circulation from the synovial structure. The plasma MP concentration was also higher when the MP was co-administered with HA than when it was administered alone. The large HA molecules may serve to trap the MP in the joint, or the HA may simply add volume to the injection, increasing the likelihood of reflux of the injectate into the subcutaneous space. The longer withdrawal associated with co-administration of HA has been previously observed with other corticosteroid intra-articular targeted therapies [15].

In order to provide practitioner guidance on how MP should optimally be used in practice, both the 95/95 tolerance limit and GCM method were compared (Table 3). The 95/95 tolerance limit is defined as a level with which there is 95% confidence that 95% of the population will fall below the threshold [6]. Practically, both methods determine the probability of violating a threshold given a risk level, in this case 5%. The primary differences between these statistical methodologies are the criteria which the data must meet for the method to be valid. For example, the 95/95 tolerance method requires

normality and a minimum number of data points of 19 [6]. The GCM method does not require a normal distribution but does require a unimodal distribution [13].

In our MP data set, the calculated threshold varied from 257 to 3160 pg/ mL, depending upon which structures were injected and which statistical method was employed to determine the threshold. The greatest discrepancy is found between the 95/95 tolerance method and the GCM (P = 0.05), with the MFT. Both methods theoretically carry the same risk of violating the threshold, but the 95/95 tolerance method is accompanied by a considerably higher threshold. Technically, the 95/95 method requires a sample size of at least 19 animals [7], and the sample size for the MFT in this study included only 10 horses. This exemplifies why it is important that the most appropriate statistical methodology for the data be employed in threshold determination. In the case of a sample size less than 19, clearly, the 95/95 tolerance level is inappropriate.

This study provides several important guidelines for veterinarians using targeted joint therapy with MP acetate in equine athletes that perform in a regulated environment. First, the use of MP in MCP, DIP and CJO joints without the concurrent administration of HA is likely to be associated with a shorter withdrawal time before a competitive event than other applications of the product. Second, caution should be used in the administration of MP to MFT joints because the plasma concentrations of MP are highly variable when MP acetate is administered into this synovial structure. Efforts to improve the accuracy of MFT injection should be made, including sedation of the patient and injection by ultrasound guidance to ensure that the entire dose is deposited within the joint pouch. Finally, DTJ and CS require a longer withdrawal for MP acetate administration than when used in other joints, likely as a result of their anatomy, and the potential for medication reflux out of the synovial structure after injection. For existing thresholds, specific withdrawal times before competition for MP acetate injection into disparate synovial structures cannot be recommended based on our findings. Additional studies are warranted to provide this information.

Authors' declaration of interests

Professor George Maylin provides drug testing services for racing jurisdictions. Professor Thomas Tobin and Dr Clara Fenger have testified frequently as experts in matters involving medication regulation.

Ethical animal research

The authors have provided confirmation that research ethics committee oversight was not required: the study was performed on material collected during clinical procedures and results were shared with owners or trainers. Owners or trainers gave consent for their animals' inclusion in this study.

Sources of funding

Funding for sample collection was provided by the North American Association of Racetrack Veterinarians, and funding for determination of drug concentrations was provided by the Equine Health and Welfare Alliance, Oklahoma Quarter Horse Racing Association, Ohio Horsemen's Benevolence and Protection Association, the Indiana Horsemen's Benevolence and Protection Association, the Ohio Harness Horsemen's Association, the Illinois Harness Horsemen's Association, the Thoroughbred Racing Association of Oklahoma and the Morrisville Auxiliary Fund.

Acknowledgements

Published as paper #486 from T Tobin and the Equine Pharmacology, Therapeutics and Toxicology Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky. The information reported in this paper is part of a project of the Kentucky Agricultural Experiment Station (KAES publication #17-16-088) and is published with the approval of the Director. The authors would like to thank Ms. Sarah Sanford for technical assistance.

Authorship

All authors contributed to study design, study execution and gave their final approval of the manuscript. J. Machin, W. Duer, C. Fenger, G. Maylin and T. Tobin also contributed to data analysis and interpretation, and preparation of the manuscript.

Manufacturers' addresses

aZoetis, Parsippany, New Jersey, USA.

bSigma Aldrich, St Louis, Missouri, USA.

cCDN Isotopes, Pointe-Claire, Quebec, Canada.

dEMD Millipore, Billerica, Massachusetts, USA.

eFisher Scientific, Fair Lawn, New Jersey, USA.

fAgilent Technologies, Palo Alto, California, USA.

References

1. Jeffcott, L.B., Rossdale, P.D., Freestone, J., Frank, C.J. and Towers-Clark, P.F. (1982) An assessment of wastage in Thoroughbred racing from conception to 4 years of age. Equine Vet. J. 14, 185-198.
2. Preston, S.A., Zimmel, D.N., Chmielewski, T.L., Trumble, T.N., Brown, M.P., Boneau, J.C. and Hernandez, J.A. (2010) Prevalence of various presale radiographic findings and association of findings with sales price in Thoroughbred yearlings sold in Kentucky. J. Am. Vet. Med. Ass. 236, 440-445.
3. Bjornsd € ottir, S., Ekman, S., Eksell, P. and Lord, P. (2004) High detail radiography and histology of the centrodistal tarsal joint of Icelandic horses aged 6 months to 6 years. Equine Vet. J. 36, 5-11
4. Anonymous. (2016). Canadian Pari-mutual Agency Elimination Guidelines 2016. Agriculture and Agri-Food Canada. Available at: http://www.agr. gc.ca/resources/prod/CMS/Internet/Common-Commun/1454071417865_ equine_elimination_guidelines_2016-eng.pdf. Accessed August 3, 2017
5. Anonymous. (2017). FEI List of Detection Times. Federation Equestre Internationale. Available at: https://inside.fei.org/system/files/2017%20FEI_detection_times.pdf. Accessed August 3, 2017
6. Anonymous (1998) European Agency for the Evaluation of Medicinal Product. Note for Guidance for the Determination of Withdrawal Periods

for Milk. European Agency for the Evaluation of Medicinal Products, Evaluation of Medicines for Veterinary Use, EMEA/CVMP/473/98 -FINAL, 11/26.

7. Knych, H.K., Harrison, L.M., Casbeer, H.C. and McKemie, D.S. (2014) Disposition of methylprednisolone acetate in plasma, urine, and synovial fluid following intra-articular administration to exercised Thoroughbred horses. J. Vet. Pharmacol. Ther. 37, 125-132
8. Rumpler, M.J., Colahan, P. and Sams, R.A. (2014) The pharmacokinetics of glycopyrrolate in Standardbred horses. J. Vet. Pharmacol. Ther. 37, 260 -268.
9. Eisenberg, R., Kudrimoti, S., Hughes, C.G., Maylin, G.A. and Tobin, T. (2014) Synthesis, purification, and chemical characterization of 20- dihydro-6 -methylprednisone, an isomeric metabolite of methylprednisolone in the horse, for use as an analytical standard. Drug Test. Anal. 6, 303-307
10. Helsel, D.R. (2012) Robust imputation and NADA (Nondetects and Data Analysis for R Software). In: Statistics for Censored Environmental Data Using Minitab_ and R, 2nd edn., Wiley and Sons, Hoboken, NJ. pp 79-98 and 297–302
11. Crawley, M.J. (2015) Statistics: An introduction using R, 2nd edn., John Wiley & Sons Ltd, Chichester, West Sussex, UK. pp 234-237.
12. Kabacoff, R.I. (2015) R in Action, 2nd edn., Manning Publications Co., Shelter, NY. pp 222-223.
13. Savage, R. (2016) Probability inequalities of the Tchebycheff (Chebyshev) type. J. Res. Natl. Bur. Stand. 65B, 211-222.
14. Clayton, H.M., Sha, H.M., Stick, J.A. and Robinson, P. (2007) 3D kinematics of the interphalangeal joints of walking and trotting horses. Vet. Comp. Orthop. Traumatol. 20, 1-7.
15. Knych, H.K., Blea, J.A., Arthur, R.M., Overly, L.R. and McIlwraith, C.W. (2016) Clearance of corticosteroids following intra-articular administration of clinical doses to racehorses. Equine Vet. Educ. 28, 140-144.

2.2.3 Pharmacokinetics of betamethasone following intra-articular injection of 15mg of BTM sodium phosphate/acetate prodrug combination into each medial femorotibial joint of Standardbred horses

Abstract: Betamethasone is an anti-inflammatory corticosteroid frequently used as an intra-articular combination of two prodrugs, Betamethasone sodium phosphate 3.15 mg/ml/mL and Betamethasone acetate 2.85 mg/ml/mL injectable suspension administered at a dose of up to 30 mg per horse. To determine the plasma pharmacokinetics and detection times of Betamethasone following BTM combination administration, we administered two 15 mg doses of BTM combination, one into each medial femorotibial joint of 8 Standardbred horses. Blood plasma samples were collected pre-administration and at selected intervals for ten days. Concentrations were determined using LC-MS/MS and the data analyzed using compartmental pharmacokinetic modeling. Plasma concentrations peaked at 30 minutes at 22 ng/ml/mL, with a mean second phase half-life of 7.07 hours until 72 hours post administration. After 72 hours, the mean plasma half-life was 123.78 hours, yielding a mean plasma concentration of 0.016 ng/ml at 240 hours post administration. These data fit a three-compartment model with absorption, with the final terminal plasma half-life of 123.78 hours. The initial rapid increase is consistent with rapid bioavailability and distribution from Betamethasone sodium phosphate, while the longer terminal phase half-life is consistent with the Betamethasone acetate suspension. Both 95/95 Tolerance and Gauss (Camp-Meidell) inequality thresholds were determined at each time point from 72 hours to 10 days. In order to accommodate medial femorotibial joint injections with 30 mg total dose of this BTM Combination, these data support a Betamethasone regulatory threshold of 0.200 ng/ml with a six-day withdrawal guideline or 0.100 ng/ml/mL with a ten-day withdrawal guideline.

Experimental: Eight standardbred horses (3 geldings, 5 mares) were used in this study. No medication was administered for a minimum of four weeks prior to the beginning of the study. A veterinarian performed the physical and lameness examinations prior. The horses were sedated with detomidine (approximately 10ug/kg, IV). The MFT joint pouch was

identified via ultrasound, and aseptic methods were used to inject 15mg of BTM combination (BetaVet) into each joint (30mg total body dose). Deposition within the joint capsules was confirmed via ultrasonography. Plasma samples were then collected out to ten days. Quantitation was performed via LC-MS/MS. Pharmacokinetics were performed via curve-stripping methodologies, and the Mean Absolute Percentage Error was 8.03%.

Conclusion: Based on our pharmacokinetic findings, we recommend an interim regulatory threshold for Betamethasone of 0.200 ng/mL with a six-day withdrawal guideline, or 0.100 ng/mL with a ten-day withdrawal guideline for use in racing regulation where bilateral MFT injections may be indicated.

Citation: Machin, J., Maylin, G., Duer, W., Fenger, C., Tobin, T. Pharmacokinetics of betamethasone following intra-articular injection of 15 mg of BTM sodium phosphate/acetate prodrug combination into each medial femorotibial joint of Standardbred horses. (2020). Submitted to Journal of Veterinary Pharmacology and Therapeutics.

Personal Contribution: Statistical and pharmacokinetic analyses of data, development of model and parameters, calculation of statistical applications (95/95 and GCM calculation confirmations, withdrawal timelines), visualization of data in figures, and writing of manuscript

Pharmacokinetics of betamethasone following intra-articular injection of 15 mg of BTM sodium phosphate/acetate prodrug combination into each medial femorotibial joint of Standardbred horses

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ABSTRACT

Betamethasone is an anti-inflammatory corticosteroid frequently used as an intra-articular combination of two prodrugs, Betamethasone sodium phosphate 3.15 mg/ml/mL and Betamethasone acetate 2.85 mg/ml/mL injectable suspension administered at a dose of up to 30 mg per horse. To determine the plasma pharmacokinetics and detection times of Betamethasone following BTM combination administration, we administered two 15 mg doses of BTM combination, one into each medial femorotibial joint of 8 Standardbred horses. Blood plasma samples were collected pre-administration and at selected intervals for ten days. Concentrations were determined using LC-MS/MS and the data analyzed using compartmental pharmacokinetic modeling. Plasma concentrations peaked at 30 minutes at 22 ng/ml/mL, with a mean second phase half-life of 7.07 hours until 72 hours post administration. After 72 hours, the mean plasma half-life was 123.78 hours, yielding a mean plasma concentration of 0.016 ng/ml at 240 hours post administration. These data fit a three-compartment model with absorption, with the final terminal plasma half-life of 123.78 hours. The initial rapid increase is consistent with rapid bioavailability and distribution from Betamethasone sodium phosphate, while the longer terminal phase half-life is consistent with the Betamethasone acetate suspension. Both 95/95 Tolerance and

Gauss (Camp-Meidell) inequality thresholds were determined at each time point from 72 hours to 10 days. In order to accommodate medial femorotibial joint injections with 30 mg total dose of this BTM Combination, these data support a Betamethasone regulatory threshold of 0.200 ng/ml with a six-day withdrawal guideline or 0.100 ng/ml/mL with a ten-day withdrawal guideline.

Key Words: Betamethasone, prodrugs, joint injection, horses, Standardbred, pharmacokinetics, regulatory thresholds, withdrawal guidelines

1/ INTRODUCTION:

Betamethasone, (11,16β)-9-Fluoro-11,17-dihydroxy-16-methyl-3,20-dioxopregna-1,4-dien-21-yl, Formula C22H29FO5, Molar Mass 392.5 g·mol−1 (BTM) is a corticosteroid widely used in equine medicine as an anti-inflammatory medication (Menendez et al., 2016). Betamethasone is administered by intra-articular injection as a combination of BTM sodium phosphate and BTM acetate for a targeted anti-inflammatory effect, as a BTM combination product, e.g., 3.15 mg/mL BTM sodium phosphate and 2.85 mg/mL BTM acetate (BetaVet®, American Regent, Shirley, NY).

Therapeutic medications such as intra-articular BTM are generally subject to regulatory restrictions on their use close to competition. The Canadian Pari-mutuel Agency (CPMA) does not publish a plasma/serum threshold but provides guidance of a 6-day withdrawal for 18 mg total intra-articular dose of a combination product (CPMA, 2016). The Federation Equestrian International (FEI) provides a detection time rather than a withdrawal guidance of 7 days for the intra-articular use of 30 mg in up to two joints (FEI, 2018). The United Stated Equestrian Federation (USEF) permits the use of BTM in competition with the submission of a medication report form (USEF, 2019). For horse racing, a common regulatory threshold in most jurisdictions in the United States for BTM is the Association of Racing Commissioners International (ARCI) guideline (ARCI, 2019), which is a 0.010 ng/mL plasma or serum threshold with a recommended seven-day withdrawal time following intra-articular administration of a single 9 mg dose of the BTM sodium phosphate/acetate combination.

Figure 2.2.3.1 Structures of Betamethasone Compounds of Interest

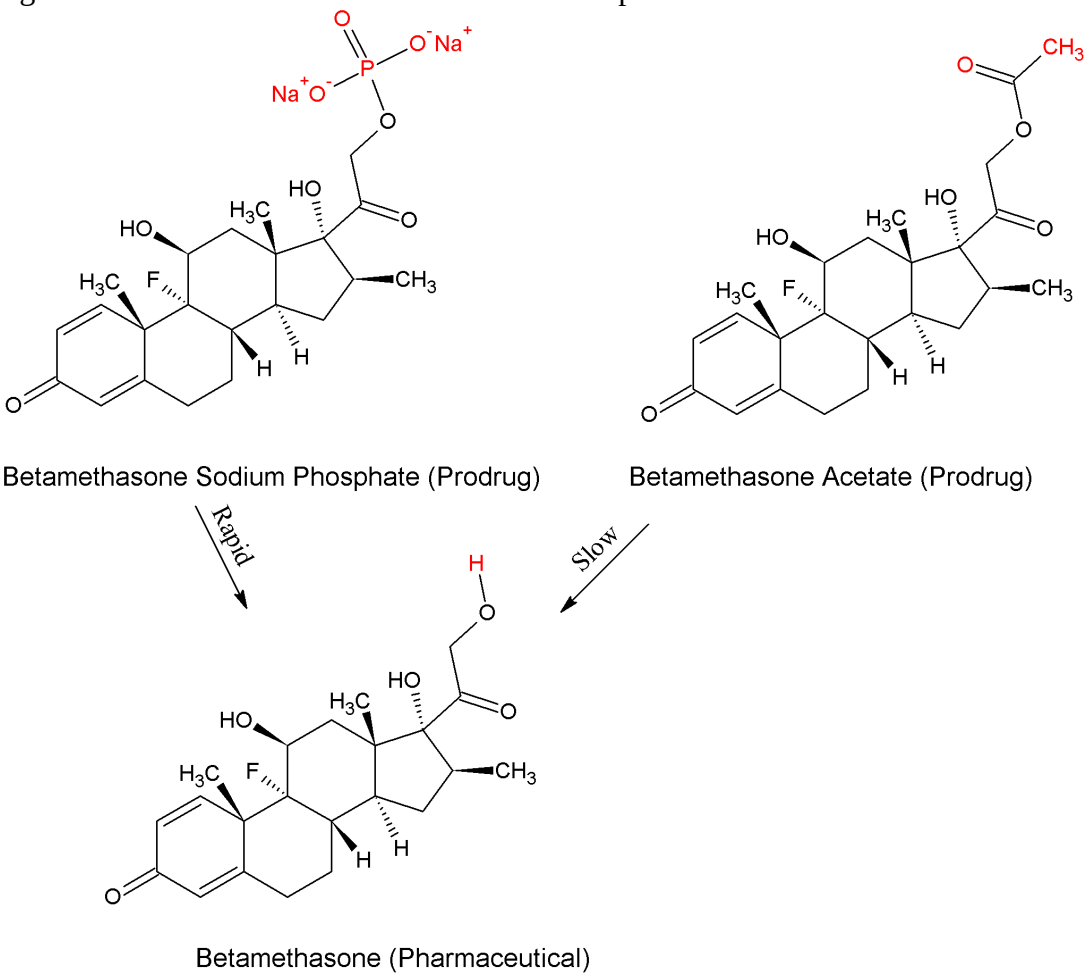

Figure 1: Structural formulae of BTM sodium phosphate *(MW 516.40), (11,16β)-9-Fluoro-11,17-dihydroxy-16-methyl-3,20-dioxopregna-1,4-dien-21-yl phosphate; BTM acetate (MW 434.50), (11,16β)-9-Fluoro-11,17-dihydroxy-16-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate; and BTM (MW 392.46), (8S,9R,10S,11S,13S,14S,16S,17R)-9-Fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro- 3H-cyclopenta[a]phenanthren-3-one.*

BetaVet® is an FDA approved BTM formulation comprised of 3.15 mg/mL of BTM sodium phosphate, and 2.85 mg/mL of BTM acetate, or 2.4 mg/mL BTM in the sodium phosphate form and 2.6 mg/mL BTM in the acetate form. The highly water-soluble BTM sodium phosphate prodrug acts to provide an initial high intra-articular concentration

of BTM while the less water soluble BTM acetate within the suspension dissolves slowly, providing a prolonged anti-inflammatory effect (Menendez et al., 2016).

The seven-day ARCI withdrawal time guideline for BTM is based on an unpublished study sponsored by the Racing Medication and Testing Consortium [RMTC], communicated in the form of an on-line monograph dated January 2019 (ARCI, 2019; RMTC, 2019). That study was conducted using administration of 9 mg of this BTM sodium phosphate/acetate combination into a single metacarpophalangeal joint. Clinically, a 9 mg dose is relatively conservative, significantly lower than the clinical doses chosen by the CPMA (18 mg), the FEI (30 mg) and published reports (Menendez et al, 2016, 30 mg). Furthermore, intra-articular injection of different anatomical structures may be associated with different withdrawal times, with targeted therapy of the stifle joint representing the most problematic procedure for withdrawal time prediction (Machin et al., 2019).

In order to provide guidance for horsemen and veterinarians operating under regulatory guidelines, a pharmacokinetic study of the clinically relevant dose of 15mg of a BTM combination product of BTM acetate and BTM sodium phosphate into each of two medial femorotibial joints, total dose 30 mg, was performed.

2/ MATERIALS AND METHODS

2.1/ Horses

Eight Standardbred horses owned by New York Drug Testing and Research Program (3 geldings, 5 mares, age (years) mean ± sd, range 8 ± 4, 4 -13, weight (kg) 454 ± 22.6, 418 - 477) were used in this study. No medication was administered for a minimum of four weeks prior to the beginning of the study. An experienced veterinary clinician (CF) performed the physical and lameness examinations prior to the study, and the horses were determined to be healthy and free of lameness. The study was conducted with the approval of the Institutional Animal Care and Use Committee of Equine Integrated Medicine, PLC (Protocol #EHWA0519).

2.2/ Drug Administration

The horses were sedated with detomidine (Dorm sedan®, Zoetis, Parsippany, NJ), approximately 10 mcg/kg, IV. The medial femorotibial joint pouch was identified by ultrasound. The skin overlying the joint pouch was aseptically prepared using povidone-iodine scrub (Betadine®, Agri laboratories Ltd., St. Joseph, MO) and rinsed with 70% isopropyl alcohol. Each medial femorotibial joint pouch was injected with 15 mg of the combination product of BTM sodium phosphate (3.15mg/mL) and BTM acetate (2.85 mg/mL) (BetaVet®, American Regent Animal Health, Shirley, NY) using aseptic technique with ultrasound guidance. The deposition of the BTM within the joint pouches was confirmed by ultrasonography after completion of each intra-articular injection (Figure 2).

2.3/ Sample Collection

Blood samples for BTM concentration determination were collected by percutaneous venipuncture at time 0, i.e., prior to tranquilization and joint injection, and at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 hours post injection. Samples were collected into heparin tubes and centrifuged at 3,000 x g. Plasma was immediately transferred into storage cryovials and stored at -20C until analysis by liquid chromatography tandem mass spectrometry (LC/MS-MS).

2.4/ Quantification of Betamethasone

The analytical procedure followed was the ISO 17025/RMTC accredited quantitative analytical procedure for BTM in place in the New York Drug Testing and Research Program. The reference standard for BTM was purchased from Sigma-Aldrich Cat. B-7005 at ≥98% purity. The reference standard for dexamethasone-d4 used as the internal standard was purchased from CDN Isotopes Cat. D-5559 at ≥96 atom %D purity. Stock solutions of BTM and dexamethasone-d4 were prepared at 1mg/mL in methanol.

Acetonitrile and methanol were purchased from EMD Millipore, and methyl tert-butyl ether and ammonium formate were purchased from Fisher Scientific. Deionized water was filtered on-site to the specification of 18.2MΩ. All reagents used were HPLC grade or better.

The BTM working solution was prepared by dilution of the 1mg/mL stock solution with methanol to the concentration of 10pg/mL. Plasma calibrators were prepared by dilution of the working standard solution with drug-free plasma to concentrations of 5, 50, 100, 1000 and 10,000 pg/mL for the first 48 h of sampling and 5, 10 and 50 pg/mL for determination of the terminal elimination portion of the curve. Quality control samples were prepared by dilution of the QC working standard solution with drug-free plasma to concentrations of 10pg/mL. Calibration curves, quality control samples, and negative control samples were prepared fresh for each quantitative assay.

Prior to analysis, 2mL of plasma was aliquoted into new, labelled test tubes. To each tube dexamethasone-d4 stock solution was added along with 5.0mL methyl tert-butyl ether. The samples were mixed by rotation for 10min, centrifuged at 2400 rpm for 5 minutes and the top layer removed and dried under nitrogen. Samples were dissolved in 50uL of methanol: acetonitrile: deionized water and a 2.5uL aliquot injected into the LC-MS/MS system, Agilent 6400 series triple quadrupole mass spectrometer coupled with an HPLC chromatography system.

The concentration of BTM was measured in plasma by LC-MS/MS using positive electrospray ionization. The mass spectrometer was operated using electrospray combined with Agilent's Jet Stream Technology. Chromatography employed a Zorbax SB-C18, 3.0 x 100mm and a linear gradient of acetonitrile (ACN) in water with a constant 5mM ammonium formate at a flow rate of 0.3mL/min. The initial ACN concentration was held at 50% for 2.75 min, ramped to 95% over 0.5 min, and held at that concentration for 1.25min before re-equilibrating for 0.5min at initial concentration.

Detection and quantification were conducted using selective reaction monitoring (SRM) of initial precursor ion for BTM (mass-to-charge ratio 393.5 m/z) and the internal standard (397.5 m/z). The response for the product ions for BTM (m/z 373, 355, and 337) and the internal standard (m/z 377) was plotted and peaks at the proper retention time integrated using MassHunter® software. MassHunter® software was used to generate calibration curves and quantitate BTM in all samples by linear regression analysis.

2.5/ Pharmacokinetic analysis:

Compartmental analysis was used for determination of pharmacokinetic parameters for intra-articular administered BTM combination with curve-stripping methodologies (Gabrielsson & Weiner, 1999). Goodness of fit and the appropriate weighting factor were selected based on visual analysis of observed versus predicted concentration graphs as well as Mean Absolute Percentage Error [MAPE], which showed the calculated predictive power to be 8.03%.

2.6/ Threshold Determination:

Data reduction methods were used for determining thresholds for Plasma BTM concentrations at 144, 168, 192, 216 and 240 h (6, 7, 8, 9 and 10 day) post injection. Population statistics were determined using a two-step process. First, all groups contained at least 2 censored (BTM concentrations below the LOQ) datapoints, so Robust Regression on Order Statistics (Helsel, 2005) were used to obtain imputed datasets as have been previously described for equine drug threshold determination (Machin et al., 2019). Second, the datasets were subjected to bootstrap analysis with 10,000 re-samplings in order to obtain an estimate of population statistics (Maindonald and Braum, 2006). The dataset at each timepoint was tested for Normality (Shapiro-Wilk, Anderson-Darling and Lillefors) and threshold determination using a 95/95 tolerance limit (Owen, 1962) and Gauss (Camp-Meidell) or GCM inequality probability equations (Savage,1961) to obtain thresholds at probability levels p= 0.05 and 0.01. The 95/95 tolerance limit corresponds to a value that

is at least above 95% of the population (assuming a normal population distribution) with a confidence of (95%). The 95/95 tolerance is calculated by:

$$xtol = m + k\,sx$$

Where xtol is the 95/95 tolerance threshold, m is the mean and sx is the standard deviation of the sample population. The k value is a tolerance limit factor equivalent to a z-score corrected for probability and sample size (Owen, 1962). The GCM is a probability inequality that provides bounds for the probability that the plasma concentration will fall within a given p-value, and requires only a unimodal distribution (Savage, 1961). All calculations for thresholds were performed using an Apple® i-MacPro® computer with macOS Catalina 10.15.1 operating system and 3 GHz 10 core Intel Xeon W10 and memory 32GB 2666MHz DDR4 system. The performance of this computer was checked by using the NIST dataset for the speed of light in air (Dorsey, 1944). The computed speed agreed to 7 significant figures with the 299792.5 km/s given by the National Institute of Standard and Technology at (https://www.itl.nist.gov/div898/handbook/datasets/MICHELSO.DAT)

3/ RESULTS

The response for the calibration curves were linear and gave correlation coefficients (R2) of 0.99 or better. Quality control sample replicates were performed (n = 7). The inter-day accuracy was 10.7% for 10pg/mL BTM. The intra-day accuracy was 3.3% for 10pg/mL BTM. The limit of detection (LOD) was 1pg/mL, and the limit of quantitation (LOQ) was 10pg/mL.

Following intra-articular administration of two 15 mg doses of BTM sodium phosphate/acetate combination into each stifle joint of eight horses (Fig. 2) the mean plasma concentrations of BTM peaked at about 22.0 ng/mL at 30 minutes post administration (Figure 3). Thereafter the plasma concentrations of BTM declined, at first rapidly with a 3.85 -hour half-life, soon followed by a slower beta-phase with a mean apparent half-life of 7.07 hours. This slower beta phase half-life held until the plasma concentration reached about 0.055 ng/mL at 72 hours post administration. Then, from 72

hours post administration on, the mean terminal plasma half-life became markedly slower, at 123.78 hours, as set forth in Figure #3 below. By ten days post administration the mean plasma concentrations of BTM were in the order of 0.016 ng/mL, which was the last time point at which plasma samples were collected.

Figure 2.2.3.2 Longitudinal Ultrasounds of Medial Femorotibial Joint Injections

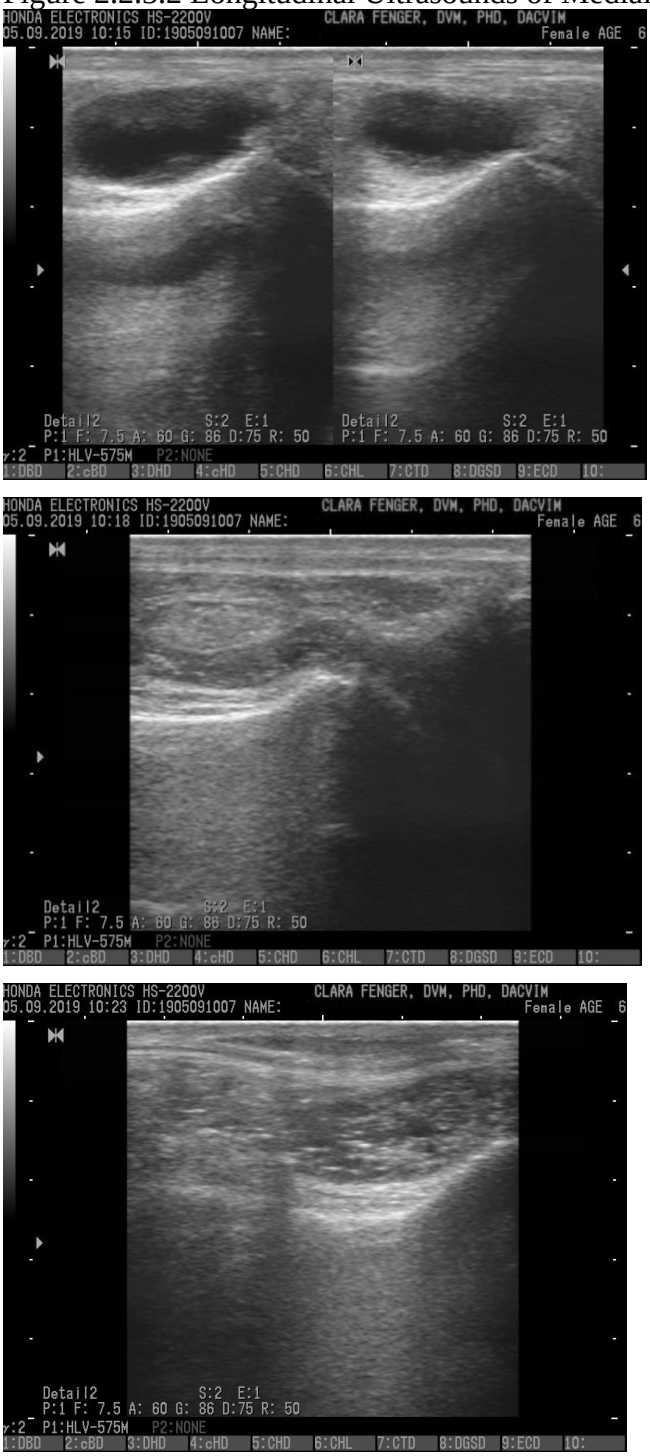

Figure 2. Longitudinal ultrasound images *of the medial femorotibial joint pouches of a horse prior to injection (a), and immediately after left (b) and right (c) medial femorotibial joint injections demonstrating increased echogenicity of the fluid after injection with BTM suspension.*

Figure 2.2.3.3 Mean Plasma Betamethasone After Injection of Two Joints

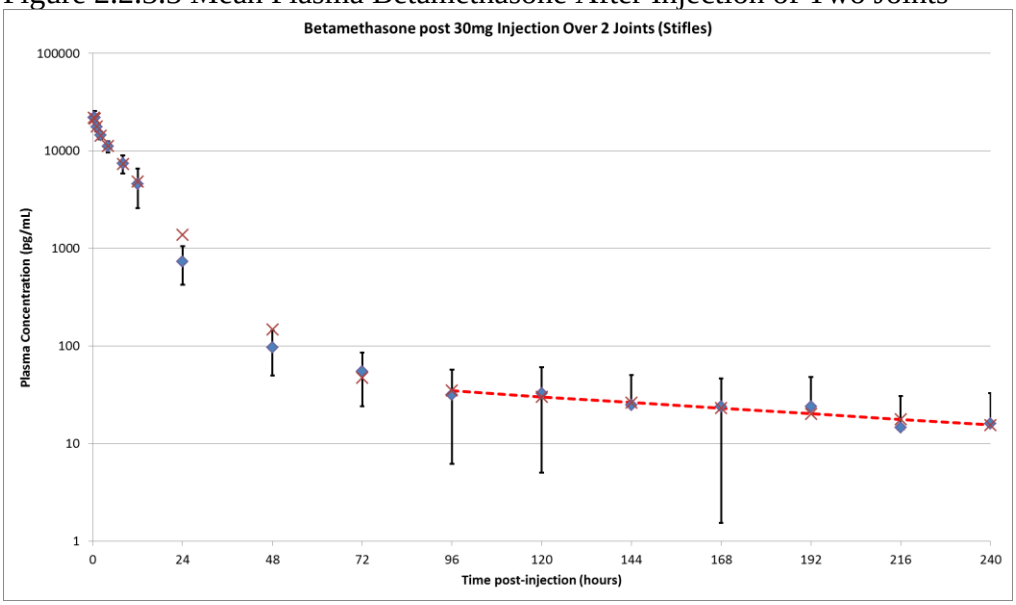

Figure 3. *The mean plasma concentrations of Betamethasone (BTM) following Intra-articular administration of two doses of 15 mg of BTM sodium phosphate and BTM acetate into each stifle joint of eight horses (◊-◊). The X--X symbols show the calculated best fit to these data points, as set forth in equation #1 below. The dashed line (---) shows the terminal elimination curve predicted by the terminal plasma half-life fitted to the days 4 to 10 mean plasma concentrations.*

The data presented in figure #3 above were best fit by a three-compartment model with absorption that is well described by the following equation, equation #1, and the relevant pharmacokinetic data are presented in Table 1 below.

$$C_t = 12.260e^{-1.65015t} + 17.177e^{-0.10671t} + 0.058e^{-0.00551t} - 29.495e^{-9.05023t}$$

Where C_t is the predicted concentration (in ng/mL) at time t (hours post-injection)

The mean terminal plasma half-life based on the day 4 to day 10 data points is 123.78 hours. The time predicted for the mean plasma BTM concentration to drop below the 0.010 ng/mL plasma threshold set forth by the RMTC following this administration and this analysis is approximately 320 hours or approaching 14 days post administration.

Table 2.2.3.1 Betamethasone Population Pharmacokinetic Parameters

Parameter	Mean ± s.d. [range]
K_a (h^{-1})	1.959 ± 1.764 [0.694 – 5.787]
K_b (h^{-1})	0.1111 $^{\pm}$ 0.0108 [0.096 – 0.125]
K_c (h^{-1})	0.0088 ± 0.0061 [0.0020 – 0.0183]
$K_{abs,I}$ (h^{-1})	8.294 $^{\pm}$ 4.865 [4.509 – 19.39]
$t_{1/2,a}$ (h)	0.575 ± 0.322 [0.120 – 0.999]
$t_{1/2,b}$ (h)	6.276 ± 0.630 [5.545 – 7.22]
$t_{1/2,c}$ (h)	129.3 ± 112.1 [37.86 – 344.8]
$t_{doubling,I}$ (h)	0.101 ± 0.039 [0.0357 – 0.154]
C_{max} (ng/mL)	24.1 ± 3.6 [19.0-2.8]
T_{max} (h)	0.375 ± 0.134 [0.25-0.5]

Table 1A Population Pharmacokinetic parameters

Table 2.2.3.2 Betamethasone Model Pharmacokinetic Parameters

Parameter	
K_a (h^{-1})	1.6502
K_b (h^{-1})	0.1067 hr^{-1}
K_c (h^{-1})	0.0055 hr^{-1}
$K_{abs,I}$ (h^{-1})	9.05 hr^{-1}
$t_{1/2,a}$ (h)	0.42 hr
$t_{1/2,b}$ (h)	6.49 hr
$t_{1/2,c}$ (h)	125.8 hr
$t_{doubling,I}$ (h)	0.0766 hr

Table 1B Model Pharmacokinetics

Table 1A & 1B: *Kinetic data as calculated based on the model equation as presented from Equation 1. K_a, K_b, and K_c are the exponential coefficients of each compartment in the model. $K_{abs,i}$ represents the initial rate of absorption as predicted in the model. Half-lives are calculated based on the exponential coefficients K_a, K_b, and K_c (and doubling time, from $K_{abs,i}$). The Area Under the Curve (AUC) was determined using a trapezoidal*

calculation, and the AUC from the final timepoint (240 hours) to infinity was estimated using the terminal half-life. Population Pharmacokinetics represent the variability of individually calculated parameters, as shown in Table 1A. Table 1B lists those parameters as determined by the model alone. If a horse were to be in the 95th percentile of slowest metabolizers in each phase, this study indicates a possible time to 0.010 ng/mL of 45 days (due to a terminal half-life of approximately 313 hours).

The Gauss(Camp-Meidell) probability curve was used to determine the likelihood of a level corresponding to the highest 5% and 1% of the population from 6 days onward. These data were considerably higher than the threshold of 0.010 ng/mL, and these values are calculated below, Table 2 and presented below along with those for the 95/95 tolerance interval, in comparison with the mean plasma concentration at each timepoint (Figure 4).

Table 2.2.3.3 Calculated Thresholds and Related Withdrawal Times for Betamethasone

Withdrawal in Days	95/95	GCM (p = .05)	GCM (p=.01)
6	0.439	0.090	0.167
7	0.265	0.079	0.143
8	0.490	0.086	0.161
9	0.097	0.026	0.034
10	0.117	0.054	0.094

Table 2 – Calculated ng/mL Thresholds and related Withdrawal Times for 15mg of Betamethasone administered as BTM sodium phosphate/acetate combination into two femorotibial joints.

DISCUSSION:

This study is the first study that identifies and presents the essentially two-phase plasma elimination pharmacokinetics of BTM administered intra-articularly to horses as a widely marketed and used clinical formulation BTM sodium phosphate/acetate prodrug combination. This BTM combination is a formulation of two different and chemically distinct prodrug forms of BTM, designed to provide an initial high concentration of free BTM at the local site of injection followed by a prolonged lower intraarticular concentration at the site of injection, and therefore an equivalent prolonged lower plasma concentration profile. The initial high concentration phase is apparently based on the rapid bioavailability of the highly water soluble BTM sodium phosphate prodrug component of the formulation. This initial phase is followed by a prolonged lower maintenance concentration of BTM, based on the slow dissolution of the less water soluble BTM acetate prodrug suspension portion of the formulation (Figure 1).

Figure 2.2.3.4 Presented Betamethasone Thresholds

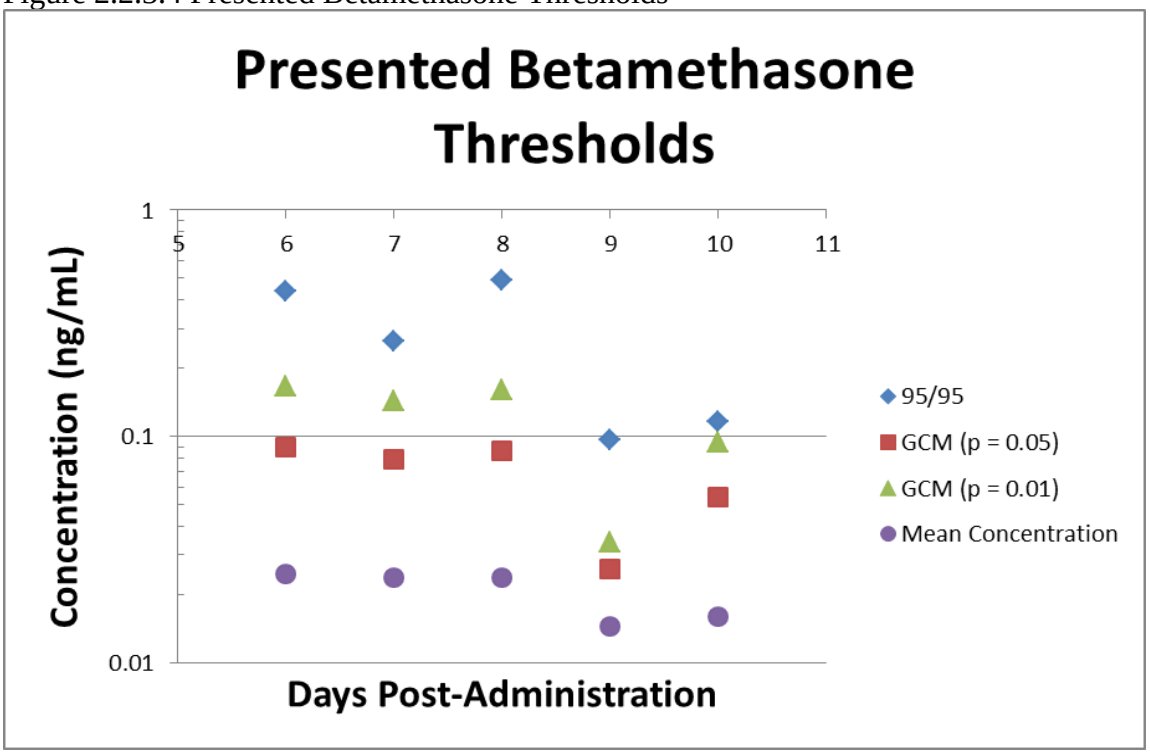

Figure 4 *shows the calculated thresholds using the two applied statistical methodologies, the RMTC 95/95 Tolerance Interval method and Gauss (Camp-Meidell). The GCM calculations are presented at probabilities of 5% and 1%. All calculations are based on plasma concentrations measured at the indicated time post-administration of 15mg of a combination of BTM sodium phosphate and BTM acetate to each of the medial femorotibial joints (30mg total dose of BTM sodium phosphate/acetate combination across two joints). Of note is that the GCM values remain below 0.100 ng/mL between days 9 and 10.*

This biphasic BTM release pattern is demonstrated in Figure 3, where the plasma concentrations of BTM peak within less than 30 minutes post-administration at 22 ± 3.7 ng/ ml. Thereafter, the plasma concentrations of BTM decline, initially rapidly, with a plasma half-life of 3.85 ± 1.52 hours, followed by a well-defined second slower beta phase with an apparent plasma half-life of 7.07 ± 1.11 hours which half-life is maintained to about 72 hours post-administration. After 72 hours post-administration the plasma half-life slows to an apparently final terminal plasma half-life of 123.78 ± 105.44 hours. This predominantly two-phase half-life elimination model is consistent with the above described two chemically distinct BTM prodrug components of the formulation. This is a classic sequence of flip-flop pharmacokinetic patterns, where the prolonged 123.78 ± 105.44 h

terminal plasma half-life of BTM is determined by the rate limiting release of BTM from the poorly soluble BTM acetate suspension portion of the BTM sodium phosphate/acetate prodrug combination formulation.

This study is in good overall quantitative agreement with the results reported by Menendez, et al, 2016, who were the first investigators to report on the plasma pharmacokinetics of a similar formulation of BTM after intra-articular administration. Menendez et al (2016) administered 30mg of a combination of 3 mg/mL BTM sodium phosphate and 3 mg/mL BTM acetate (Celestone Soluspan®, Merck &Co, Inc., Whitehouse Station, NJ) intra-articularly, 15 mg into one randomly assigned tarsometarsal joint and the second 15 mg dose into the ipsilateral metatarsophalangeal joint. Following these administrations, plasma concentrations of BTM peaked at about 26ng/mL at 48 minutes post administration and thereafter declined with an apparent plasma half- life of 9.22 hours, a fully comparable but fractionally longer half-life than the second rapid elimination phase plasma half-life (from Kb) of 7.07 hours presented in the data of Figure 2 above, consistent with the slightly higher proportion of the poorly soluble BTM acetate fraction.

Figure 2.2.3.5 Three Compartment Interaction Model for IA Betamethasone

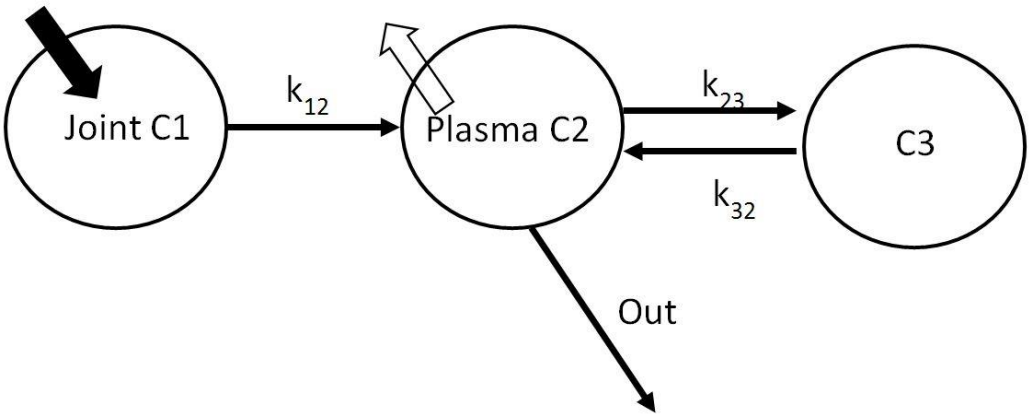

Figure 5*: Three compartment interaction model of intra-articular injection of bilateral stifles with a total dose of 30 mg combination BTM sodium phosphate and BTM acetate. The large black arrow indicates administration of 15 mg BTM combination into the joint compartment(s). The symbol* k_{12} *indicates the essentially unidirectional diffusion of BTM*

from the injected joint to the plasma compartment C2, the sampling site for BTM analysis and this compartment in equilibrium with C3.

Menendez, et al (2016) reported their last plasma detection of BTM at 64 hours post administration, presumably at or about their LOQ of 0.050 ng/mL. Because of their inability to detect BTM concentrations lower than 0.050 ng/mL, Menendez, et al did not identify the final slow terminal plasma half-life of BTM reported in our experiments, the final terminal mean plasma half-life of BTM being 123.78 hours in our results (that is, what our data suggests to be the true terminal half-life). However, within the limits of the available methodology there is good overall agreement between the Menendez data as presented and the data presented in our Figure 3 above.

A more recent communication from Knych et al, 2018, reports plasma concentrations of BTM following intra-articular administration of BTM combination at the product label single joint dose of 9 mg. We also note that this 9 mg label dose is a conservative dose, as shown by the fact that the American Regents Animal Health information notes that "BTM sodium phosphate/acetate combination "may be administered concurrently in up to two joints per horse" for a total dose of 18 mg. Similarly, Menendez, et al in their study on Intra-articular administration of a similar formulation of BTM as Celestone Soluspan® administered a dose of 15 mg/joint into each of two joints, for a total I/A dose of 30mg in their pharmacokinetic study, as presented above.

Knych et al. studied the pharmacokinetics of a 9 mg right antebrachiocarpal joint intra-articular dose of the same BTM combination (BetaVet®) to 12 horses. In these experiments, Knych et al reported peak plasma concentrations of BTM of 3.97 ng/mL at 1.45 hrs post administration, a somewhat slower time to peak concentration than in our experiments. However, these peak plasma concentrations reported by Knych et al and those reported in the current data are in broad agreement. The small differences may be attributed to the higher total dose used in our study, the injection into two joints, and/or the anatomy of the different joints injected. Previous studies have demonstrated that plasma concentrations of methylprednisolone vary depending upon the anatomical structure

injected (Machin et al., 2019), and it is likely that other pharmacokinetic parameters vary as well.

The early phase apparent plasma half-life identified in our study, (Figure 3, Table 1) is in good agreement with the 7.48 hr. plasma half-life reported by Knych et al. This early rapid phase half-life is presumably driven by the more readily bioavailable BTM sodium phosphate in the BTM sodium phosphate/acetate prodrug combination formulation. However, the Limit of Quantitation (LOQ) of the analytical method used in the Knych study was, at 0.025 ng/mL in plasma, insufficiently sensitive to allow detection of the much longer half-life secondary elimination phase of BTM following administration of this relatively low 9 mg dose of their BTM combination formulation. Knych, et al did not identify the longer second phase terminal elimination half-life of BTM that has been clearly identified in our higher dose study, however, this is not unexpected because the analytical method used in the Knych study had an LOQ of 0.025 ng/mL, as compared to the LOQ of 0.010 ng/mL in our study. Other than this difference in terminal elimination, there is good agreement overall between the Knych BTM data and the data of figure #3. In particular, the higher dose divided between two joints may account for the earlier Tmax and also the higher Cmax reported in this study. Further, the terminal elimination of 123.78 ± 105.44 h identified in this study could not have been identified using the lower dose and less sensitive analytical procedure in the previous study.

The purpose of this study was to identify thresholds for BTM after the use of a BTM combination product following intra-articular administration into bilateral femorotibial joints at a clinically relevant dose. Therefore, these data were used to compare different methods of threshold calculation, both the 95/95 threshold and also the Gauss Camp-Meidell thresholds from each sampling time point from 72 hours on, as presented in table 2 and figure 4. The 95/95 threshold was consistently higher than both the GCM ($p=0.01$ and 0.05). The 95/95 tolerance method is defined as a level that provides 95% confidence that 95% of the population will fall below the threshold. As has been previously shown (Machin et al., 2019), the 95/95 tolerance method is inaccurate when employed with

small sample sizes. The GCM (P=0.01) threshold likely provides a better estimate of a true threshold for our data.

These data show variability in the calculated thresholds from day-to-day post administration. Each individual horse exhibits a progressive decline from joint injection to 96 h post-administration. After the 96-h point, the plasma BTM concentrations for each horse increase and decrease from day to day, with an overall downward trend. As the terminal elimination of the drug is likely due to the rate limiting step of dissolution of the BTM acetate component, this is attributable to individual variability, which may include the activity of the animal with mechanical disruption of the BTM acetate particles, changes in blood supply associated with exercise, or even temperature, affecting the solubility of the drug. In 2 horses that were briefly exercised after the 72-h plasma sample, one horse had a four-fold higher BTM plasma concentration after light exercise (data not shown), indicating that even light exercise may affect the plasma concentration of BTM. In our study, the 6-day BTM plasma concentrations using the GCM (p=0.01) threshold fell below 0.200 ng/mL and both the 9- and 10-day GCM (P=0.01) fell below 0.100 ng/mL, indicating that 0.200 ng/mL is sufficient for a 6-day threshold and 0.100 ng/mL is sufficient for a 10-day threshold for bilateral femorotibial joint injection with 15 mg BTM combination.

Extrapolation of the elimination equation estimates 14 days for the average horse injected with bilateral medial femorotibial joints with 15 mg each of BTM combination to drop below the ARCI threshold of 0.010 ng/mL. However, this is the average horse, and a horse that may metabolize intra-articular BTM combination slowly may require as much as 45 days to drop below the 0.010 ng/mL ARCI regulatory threshold.

5/ **CONCLUSIONS**

Intra-articular administration of 30 mg of BTM combination as a BTM sodium phosphate/acetate prodrug combination as two 15 mg/joint intra-articular stifle joint injections yielded a rapid initial peak plasma concentration of 22 ± 3.7 ng/mL of BTM at about 30 minutes post administration. Thereafter plasma concentrations of BTM fell rapidly, following a three-compartment elimination pharmacokinetic model. The initial plasma half-life was brief, 3.85 hours, followed by a longer second phase half-life of 7.07 hours. This second phase half-life was maintained out to about 72 hours, at which point

the mean plasma concentration of BTM were on the order of 0.055 ng/mL. Thereafter, plasma concentrations of BTM fell much more slowly, with an apparent mean terminal plasm half-life of about 123.78 hours, to reach a mean plasma concentration of 0.016 ng/mL at ten days post administration. This terminal elimination half-life was used to predict that most horses would fall below the in-place threshold in the United States of 0.010 ng/mL at 14 days after stifle injection. This is the first published report describing the critically important prolonged terminal plasma half-life of BTM following its intra-articular administration as a BTM combination formulation in horses, providing guidance as to the likely detection times for BTM following its administration at clinically useful intraarticular doses of BTM sodium phosphate/acetate combinations to racing horses. Based on the available data we calculated the GCM and 95/95 regulatory thresholds at each time point from 6 days on and now suggest interim BTM regulatory thresholds of 0.200 ng/mL with a six-day withdrawal guideline or 0.100 ng/mL with a ten-day withdrawal guideline for use in racing regulation where bilateral MFT joint injection may be indicated.

ACKNOWLEDGEMENTS:

This research has been made possible by support from The Equine Health and Welfare Alliance, Inc, Versailles, Kentucky, and the United States Trotting Association, Columbus, OH. Other support that has made this work possible includes the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch Program under project KY014051 Accession Number 1010609 and also by grants from the USDA Agriculture Research Service Specific Cooperative Agreement #58-6401-2-0025 for Forage-Animal Production Research, the Kentucky Department of Agriculture and the Kentucky Thoroughbred Association Foundation and by support for the Kentucky Agricultural Experiment Station as provided by the National Institute of Food and Agriculture (NIFA) and the Commonwealth of Kentucky. Published as paper #502 from T. Tobin and the Equine Pharmacology, Therapeutics and Toxicology Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky.

CONFLICT OF INTEREST

The authors recognize no conflict of interest in publishing this paper.

AUTHORS' CONTRIBUTIONS

All authors JM, GM, WD, CF, and TT contributed to the design, data analysis and final data interpretations. CF performed the intra-articular injections and GM performed the sample analyses. WD performed and supplied the RROS data where needed and calculated GCM values. JM and TT performed the pharmacokinetic analysis and contributed to the overall interpretations. All authors contributed to the final format of the paper, and all have read and approved the final submitted manuscript.

REFERENCES:

1/ Menendez MI, Phelps MA, Bertone AL. 2016. Pharmacokinetics of intra-articular betamethasone sodium phosphate and betamethasone acetate and endogenous hydrocortisone suppression in exercising horses. J Vet Pharmacol Ther. Feb;39(1):22-6. doi: 10.1111/jvp.12229. Epub 2015 Apr 3.

2/ Canadian pari-mutuel Agency. 2016. http://www.agr.gc.ca/resources/prod/CMS/Internet/Common-Commun/1454071417865_equine_elimination_guidelines_2016-eng.pdf, accessed September 25, 2019.

3/ Federation Equestrian Internationale. 2018. https://inside.fei.org/system/files/FEI%20Detection%20Times%202018_0.pdf, accessed September 25, 2019.

4/ United States Equestrian Federation. 2019. https://www.usef.org/forms-pubs/2Zp2C_YKs4s/2019-equine-drugs-medications, accessed September 25, 2019.

5/ Association of Racing Commissioners International. 2019. http://arci.com/wp-content/uploads/2019/01/2019_01_CTS_V4_1.pdf, accessed September 25, 2019.

6/ Racing Medication and Testing Consortium. 2019. https://rmtcnet.com/wp-content/uploads/RMTC-Betamethasone-Monograph-Jan-2019.pdf, accessed September 25, 2019.

7/ Machin, J., Duer, W., Maylin, G., Fenger, C., Wilson, D., Ivey, M., ... & Tobin, T. (2019). Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: A field study. Equine veterinary journal, 51(3), 343-348.

8/ Gabrielsson JL, & Weiner DL. 1999. Methodology for pharmacokinetic/ pharmacodynamic data analysis. Pharmaceutical science & technology today, 2(6), 244-252.

9/ Helsel, D. R. (2005). Nondetects and data analysis. Statistics for censored environmental data. Wiley-Interscience.

10/ Maindonald, J., & Braun, J. (2006). Data analysis and graphics using R: an example-based approach (Vol. 10). Cambridge University Press.

11/ Owen, D. B. (1962). Handbook of statistical tables (No. R HA48 O9).

12/ Savage, I. R. (1961). Probability inequalities of the Tchebycheff type. Journal of Research of the National Bureau of Standards-B. Mathematics and Mathematical Physics B, 65(3), 211-222.

13/ Dorsey, N. E. (1944). The velocity of light. Transactions of the American Philosophical Society, 34(1), 1-110.

14/ Knych HK, Stanley SD, Harrison LM, Mckemie DS. 2017. Pharmacokinetics of betamethasone in plasma, urine, and synovial fluid following intra-articular administration to exercised thoroughbred horses. Drug Test Anal. Sep;9(9):1385-1391. doi: 10.1002/dta.2170. Epub 2017 Mar 7.

2.3 Environmental Sources

2.3.1 Review and Analysis of an Interim Screening Limit of Detection for Naproxen in Post-Race Samples

Abstract: Starting in August 2015 Thoroughbred racing in Charles Town, West Virginia experienced a sequence of intermittent low concentration Naproxen identifications from 6.3 to 161 ng/ml of plasma (27.3 to 699 nM). These identifications were ongoing, indicating the horsemen were unaware of their origins. Naproxen is administered orally to horses at substantial doses and is chemically stable in the environment. These identifications are therefore most likely associated with exposure of these horses to environmental traces of Naproxen. Given the low concentrations of these identifications, we were asked to identify a Screening Limit of Detection (SLOD) below which these trace level Naproxen identifications would not be reported. Review of the data set suggested an SLOD of 200 ng/ml, while outlier analysis suggested an 'extreme' outlier level at 247 ng/ml, which figure was rounded up to 250 ng/ml Naproxen or 1.09 uM. This proposed SLOD is in good agreement with other US regulatory thresholds for therapeutic medications and this Interim Screening Limit of Detection was presented for review

Conclusion: Using statistical analyses, including outlier analysis of apparently 'innocent' positives that were called out of Charles Town racetrack after switching testing locations, a suggested interim screening limit of detection was proposed at 250 ng/mL for naproxen.

Citation: Machin, J., Brewer, K., Catignani, M., Shults, T. F., Fenger, C., Maylin, G. A., & Tobin, T. (2020). An interim screening limit of detection for naproxen in equine plasma: a review and analysis. Comparative Exercise Physiology, 16(2), 153-160.

Personal Contribution: Statistical and pharmacokinetic analysis and model development, collation of outside data points for meta-analysis, development of environmental contamination model and safety factor comparison, and writing of manuscript

An interim screening limit of detection for naproxen in equine plasma: a review and analysis

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Abstract

Starting in August 2015 Thoroughbred racing in Charles Town, West Virginia experienced a sequence of intermittent low concentration Naproxen identifications from 6.3 to 161 ng/ml of plasma (27.3 to 699 nM). These identifications were ongoing, indicating the horsemen were unaware of their origins. Naproxen is administered orally to horses at substantial doses and is chemically stable in the environment. These identifications are therefore most likely associated with exposure of these horses to environmental traces of Naproxen. Given the low concentrations of these identifications, we were asked to identify a Screening Limit of Detection (SLOD) below which these trace level Naproxen identifications would not be reported. Review of the data set suggested an SLOD of 200 ng/ml, while outlier analysis suggested an 'extreme' outlier level at 247 ng/ml, which figure was rounded up to 250 ng/ml Naproxen or 1.09 uM. This proposed SLOD is in good agreement with other US regulatory thresholds for therapeutic
medications and this Interim Screening Limit of Detection was presented for review.

Keywords: naproxen, horseracing, environmental contaminant, clinical practice, screening limit of detection

Introduction

Naproxen ((+)-(S)-2-(6-Methoxynaphthalen-2-yl)propanoic acid, C14H14O3, 230.259 g/mol, (Figure 1) is a non-steroidal anti-inflammatory drug (NSAID) widely used in human and equine therapeutic and sports medicines (Tobin, 1981). The dose of Naproxen is large, 500 mg in humans twice a day, 2 to 5 g in equines, once or twice per day (Lees and Higgins, 1985). Naproxen also plays an important role in both human and equine sports training, used both to combat inflammation and exercise-related pain. It has been shown to be effective in treating exercise-induced dysfunction, muscle injury, and soreness (Dudley et al., 1997). Naproxen has also been shown to be beneficial for aging individuals undergoing resistance training by attenuating muscle injury, strength loss, and soreness following eccentric muscle actions (Baldwin et al., 2001). Next, Naproxen has been shown to be beneficial when used for brief periods of time for short-term recovery of muscle function after exercise-induced muscle injuries (Lanier, 2003). In World Anti-Doping Agency (WADA) regulated human sports, there are no restrictions on the presence/use of Naproxen during competition (WADA, 2019) but racehorses are not permitted to compete under the influence of pharmacologic levels of most medications. Despite this, Naproxen may be administered outside of competition for therapeutic purposes. It is orally administered and eliminated at high concentrations in urine resulting in a significant environmental presence that is unavoidable in a treatment stall.

Figure 2.3.1.1 Chemical structure of Naproxen

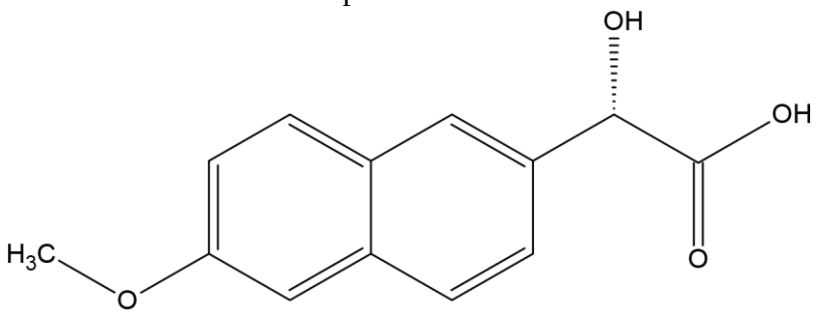

Figure 1 *Chemical structure of Naproxen*

Naproxen is stable in the environment, persisting for at least a year (Zuccato et al., 2000), which means that although administration ceases, a horse remains exposed to

significant amounts of environmental Naproxen from the treatment stall. These amounts are sufficient to give rise to trace level plasma identifications as long as the animal remains in the treatment stall. Additionally, the time for the animal to reach an undetectable plasma/urine level after removal from the treatment stall is undefined and largely dependent on the sensitivity of the drug testing in place (Tobin et al., 2013). Adding to this, there is a significant amount of background contamination as raw drinking water often contains small concentrations of Naproxen. In the US 70% of tested Raw Drinking water facilities measured between 16 and 44 ng/l of Naproxen in 2008 (Snyder, 2008). The amounts of environmental Naproxen to which such horses are exposed are not pharmacologically significant, being generally less than 2 μg Naproxen per day. While these amounts are much lower than any dose to reach an effective plasma level, estimated by Toutain et al. (2002a, b) to be approximately 10 μg/ml, their presence in the environment should be noted as a potential source and variable.

A second potential source of environmental Naproxen is humans that are taking Naproxen. Humans may then inadvertently transfer Naproxen to the horse or its environment and thereby give rise to trace level Naproxen identifications, as has been shown for humans on Tramadol (British Horseracing Authority, 2015). In humans, ~80% of Naproxen is excreted as a conjugated metabolite, with 51% of the total excretion being a simple acyl-glucuronidated form (Davies and Anderson, 1997). In horses, less than 1% of the total compound excreted remains unchanged, with ~40% requiring only hydrolysis of glucuronide to be recovered as the parent compound (Soma et al., 1995). Given a full course of Naproxen (5g BID for 7 days) has passed through a horse in a single stall, a treatment stall could contain up to approximately 28 g of Naproxen. Similarly, the dose of Naproxen for humans is approximately 1 g total per day, indicating a total potential daily contamination from humans of up to 200 mg parent Naproxen and 510 mg Naproxen acylglucuronide.

Given these circumstances, it should come as no surprise that low-concentration trace level identifications of Naproxen may occur associated with therapeutic use of this medication, as was first suggested by research on orally administered Naproxen performed by our Canadian colleagues (Stevenson et al., 1997), which data we have digitized, recalculated, replotted and reanalyzed in Figure 2 below. We note in particular the fact that

at 80 hours post-Naproxen administration the plasma concentrations of Naproxen in these horses stopped declining and in fact in one case increased after 100 hours post the last dose. In addition, all of these post-80-hour plasma concentrations of Naproxen are significantly above the 161 ng/ml and lower Charles Town identifications. Consistent with our re-analysis and interpretation of these Canadian data, later research in Sweden (Wennerlund et al., 2000) and Louisiana (Barker, 2008) has made clear that this very slow final 'clearance' of Naproxen from these horses is most likely related to environmental Naproxen retained in the treatment stall.

Figure 2.3.1.2 Pharmacokinetic Analysis of Reported Naproxen Concentrations

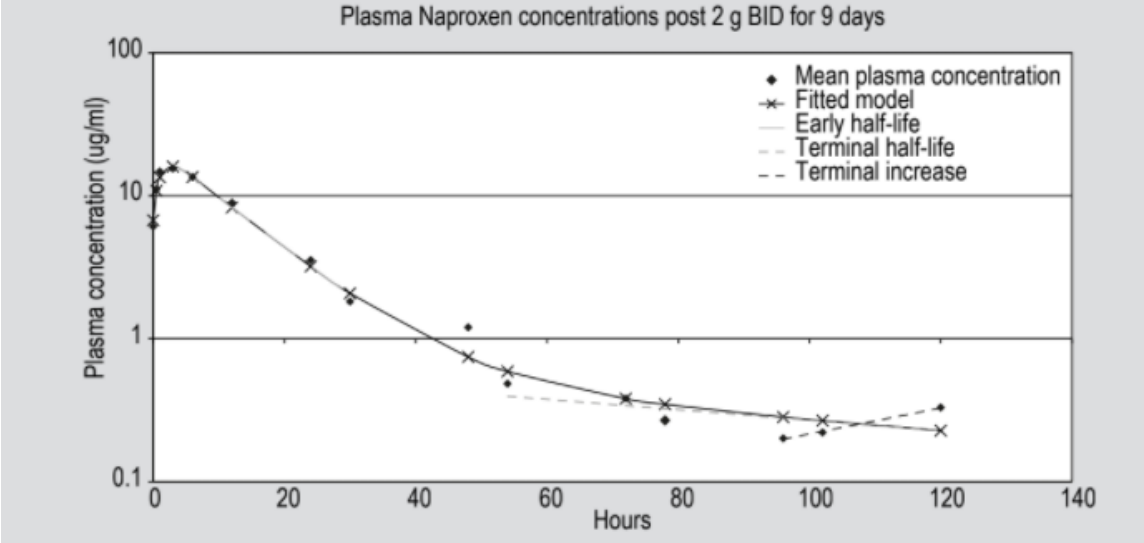

Figure 2. *Pharmacokinetic analysis of mean plasma Naproxen concentrations following 2 g twice daily for 9 days to four horses. In this experiment, published Canadian plasma data were recalculated, and replotted, curve fitted and reanalyzed as set forth in methods. The overall fit to the data includes an initial plasma half-life of about 8.8 hours, followed by an approaching tenfold slower rate of decline from about 80 hours post administration. Additionally, and unusually, there was an overall 64% increase in the mean plasma concentrations following 96 hours, best explained by significant ongoing exposure of these horses to Naproxen. Replotting, reanalysis and reinterpretation of plasma Naproxen data from Stevenson et al. (1997).*

With regard to the matter of Naproxen being retained in the treatment stall, our Swedish colleagues (Wennerlund et al., 2000) emphasize the extreme risk of contamination associated with Naproxen use, as follows: 'An untreated horse put in a box contaminated with Naproxen, could not, as far as the urine concentrations were concerned, be distinguished from a horse treated with Naproxen' and 'Consequently, it is strongly suggested that competition/racing horses should be treated in separate treatment boxes and then removed. Treatment boxes should only be used for horses undergoing treatment. *A box cannot be considered clean even if the crib has been scrubbed* [emphasis added].'

This current sequence of Charles Town Naproxen identifications began in August 2015 when Industrial Laboratories of Denver, Colorado took over the Charles Town equine

drug-testing contract. Previously, no trace level identifications of Naproxen had been reported at Charles Town Races, apparently because a plasma regulatory threshold of 1000 ng/ml in place in the East Coast Mid-Atlantic States was also being used in Charles Town's testing. However, soon after the Colorado laboratory took over testing, the first trace level identification of Naproxen at 31 ng/ml was reported, on August 15th, 2015. This sequence of sporadic, low concentration Naproxen identifications then continued up until March 2017, as presented in Table 1, below.

Table 2.3.1.1 Naproxen Positives at Charles Town

Date	Amount (ng/ml)	Laboratory
January to July 2014	no Naproxen positives	Dalare
August 2014-June 2015	no Naproxen positives	Truesdail
August 15, 2015	31	Industrial
September 11, 2015	4,200	Industrial
December 18, 2015	23.2	listed as Truesdail?
December 18, 2015	37.7	Industrial
January 8, 2016	161	Industrial
July 9, 2016	26.4	Industrial
November 4, 2016	18.0	Industrial
November 18, 2016	6.3	Industrial
November 26, 2016	34.1	Industrial
December 7, 2016	43.7	Industrial
January 19, 2017	96.6	Industrial
January 27, 2017	86.9	Industrial
March 10, 2017	11.8	Industrial
March 11, 2017	19.5	Industrial

[1] No Naproxen positives were called during testing by Dalare Laboratories, first half 2014, or by Truesdail Laboratories, second half of 2014 and first half of 2015. Then, after Industrial Laboratories commenced testing, the indicated sequence of Naproxen 'positives' commenced.
[2] Note, at the time of submission, there have been several rescinded calls for Naproxen from Charles Town. The above data does not reflect this new information, just the reported analytical findings.

Table 1. *This table lists our best analysis of the Naproxen 'positives' called in Charles Town since January 2014.*[1,2]

Review of Table 1 shows a total of about 14 Naproxen identifications over an approximately 21-month period. With the exception of one early 4,200 ng/ml identification, all identifications were at or below 161 ng/ml with the majority below 50 ng/ml. Simply put, with one early exception, this presents as a sequence of low-concentration identifications with no possibility of pharmacological effect. Furthermore, this sequence of identifications continued despite the fact that Charles Town horsemen were aware that these trace concentrations of Naproxen were being called 'positive', a

pattern consistent with the horsemen involved in these matters being unaware of how to prevent these identifications.

On the other hand, the single 4,200 ng/ml identification occurring early in this sequence in September 2015 is consistent with an intentional administration, the Horseman then becoming aware that these concentrations of Naproxen were being detected and reported, and no further identifications above 161 ng/ml have since been reported. This 4,200 ng/ml identification was therefore attributed to an intentional administration, and not to being of unknown environmental origin.

Identifying a Screening Limit of Detection (SLOD) for an environmental substance is by definition a 'bottom-up' analysis, as follows. Since the data points are the result of random environmental exposure of unknown origin, the only data on which such a SLOD can be based are actual field data that can reasonably be attributed to an environmental source. One reviews the data, performs appropriate statistical analyses and then identifies an appropriate SLOD, namely a screening limit of detection which effectively eliminates the administrative problem of irrelevant low concentration identifications that are difficult or impossible for horsemen, or indeed the industry in general, to control and also are of no pharmacological or regulatory significance, as has been determined for a number of trace level identifications of methamphetamine and dextrorphan (Brewer et al., 2016; Machin et al., 2017).

Materials and methods

Data were collected as reported and tabulated in Table 1 before being statistically analyzed. Outliers were determined using Tukey's method. A second statistical analysis was constructed without the use of the 4,200 ng/ml datapoint as well and is noted in the Results section. The Naproxen plasma data presented in Figure 2 were digitized, re-analyzed and replotted from Stevenson et al. (1997) as follows. The presented data points were pooled, and the mean values obtained at each time point calculated. The mean data points were then fitted by standard curve fitting procedures using a two-compartment model with absorption. The equation that best fit these data points was Equation 1:

Equation 1

$$C_t = 22.05538^{-0.08768t} + 0.62269^{-0.00842t} - 16.05961^{-0.77502t}$$

The early 8.8-hour plasma half-life in Figure 2 was calculated based on the time points from 3 to 30 hours, and the longer ‘apparent’ terminal plasma half-life of 82.3 hours was calculated on the 54-to-120-hour time points and the late ‘apparent’ doubling time on the 96-to-120-hour time points, respectively.

Figure 2.3.1.3 RMTC Safety Factors vs Effective Plasma Concentration

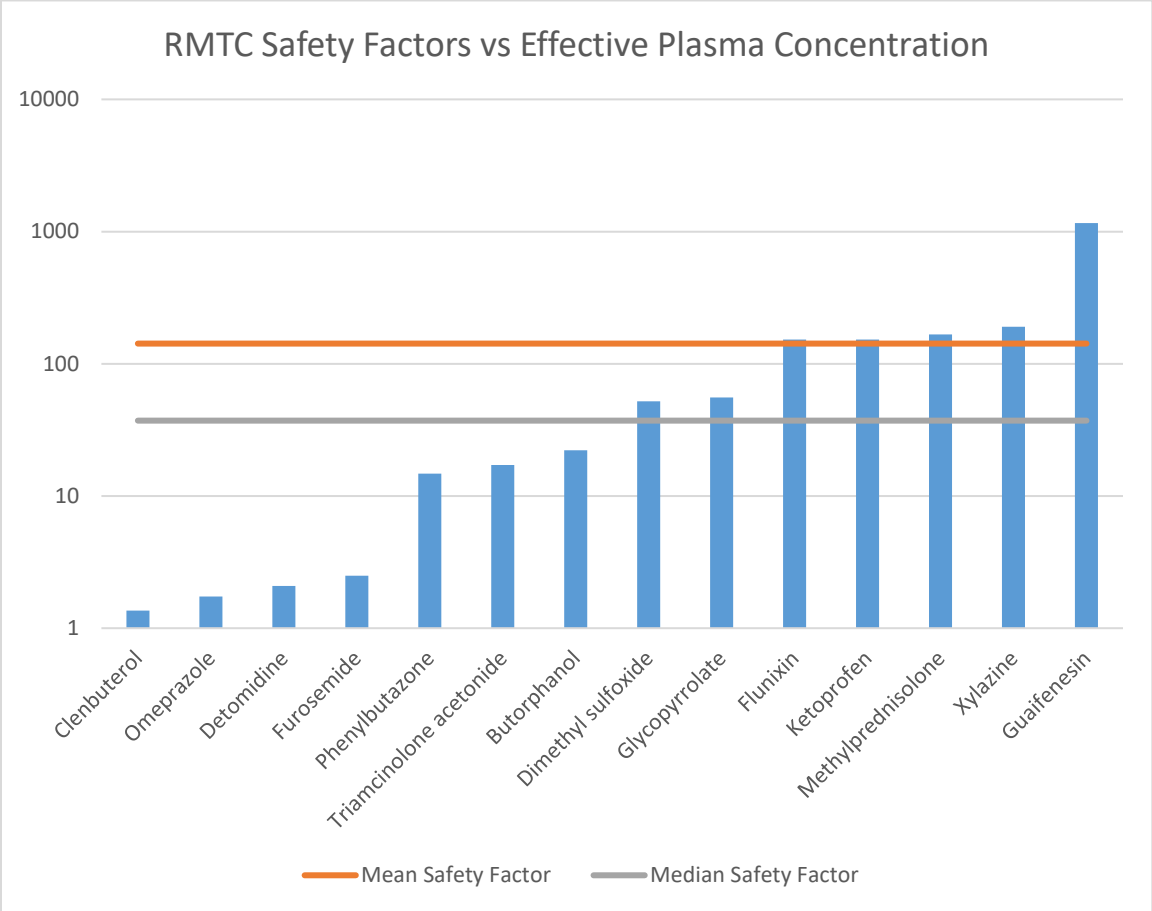

Figure 3.2.1 shows the safety factors of various compounds compared to their EPC as calculated based on the pharmacological parameters reported by Toutain.

The other factor to take into account – the health and welfare of the animal – is well-characterized by the opposite end of the spectrum, guaifenesin. Guaifenesin is therapeutically used in both humans and horses as an expectorant to help clear mucus from the airways in the lungs. However, it also acts as a centrally acting skeletal muscle relaxant. As such, it may 'mask' an animal's health issues if it is not truly sound and allow a horse to race whenever it has a higher potential for catastrophic injury. This is in turn an argument for the much higher safety factor taken for guaifenesin when comparing the regulatory threshold (12 ng/mL) to the EPC (13,889 ng/mL).

Table 2.3.1.2 Statistical Summary of Naproxen Positives

	Entire dataset	Excluding 4,200 datapoint
Mean	342.55	45.82
Standard deviation	1,111.05	43.76
Minimum	6.3	6.3
Q1	20.43	19.5
Median	32.55	31
Q3	76.18	43.7
Maximum	4,200	161
Range	4,193.7	154.7
Inter-quartile range	55.7	24.2
# Mild outliers	1	2
# Extreme outliers	1	1
1/10,000 threshold	4,474.53	208.58

[1] The 1/10,000 threshold is the cut-off to allow for a false-positive detection rate of 1/10,000, within this population. All values are represented in ng/ml.

Table 2. Statistical summary of the dataset inclusive and exclusive to the original extreme outlier of 4,200 ng/mL.[1]

Figure 2.3.1.4 Outlier Analysis of Naproxen Positives

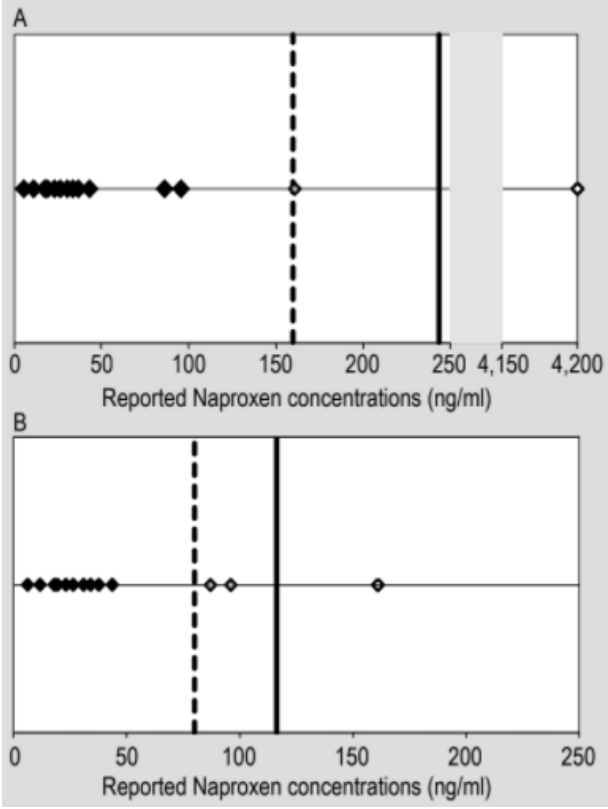

Figure 3. *(A) Outlier analysis of the data of Table 1. (B) Outlier analysis of the data after removal of the 4,200 ng/ml point. Outlier fences are shown for 'mild' (dotted line) and 'extreme' outliers (solid line).*

Results

The data set was initially inspected as an entire set, with its statistical summary indicated in Table 2. Using the entire dataset, the 5-number summary of the data was calculated, and outliers were determined one-directionally using the Tukey method (Q3 + 1.5×IQR for mild outliers and Q3 + 3.0×IQR for 'extreme' outliers) (Figure 3A). These results yielded 'fences' at 160 ng/ml for 'mild' outliers and 243 ng/ml for extreme outliers, which figure we rounded up to 250 ng/ml. Upon analysis via Tukey's method for outliers (and with reasonable belief that it was not due to environmental contamination as the others potentially were), the highest datapoint of 4,200 ng/ml was removed from the set and a secondary statistical analysis was performed on this new dataset (Table 2, Figure 3B). Although noticeably different, it still contains an extreme outlier and two moderate outliers, leaving open the question of the genuine distribution to be expected. 1/10,000 one-sided probability puts a likely point at 208 ng/ml. Due to the small sample size for this determination, the historical threshold of 1000 ng/ml, and the known ability for Naproxen to persist and contaminate environments 250 ng/ml seems appropriate as a threshold until further information is obtained.

Discussion

In contrast to human sports, in competitive events involving animals, it is critical that they not compete under the pharmacologic influence of pain mitigating substances (Toutain and Lassourd, 2002b). At the same time, regulation of medication must take into consideration low levels of substances due to environmental transfer. This is addressed in human sports by established decision limits for threshold substances (WADA, 2017). Few such screening limits/decision limits for environmental substances exist in equine sports.

Review of the data in Table 1 with the assumption that all data points at or below 161 ng/ml are identifications associated with environmental origin demonstrates that the SLOD must be greater than 161 ng/ml, leading to a first approximation of 200 ng/ml. Next, we performed a Tukey outlier analysis on the entire data set, which suggested that the 161 ng/ml identification was a 'mild' outlier and the 4,200 ng/ml value an 'extreme' outlier, consistent with the results of our inspection and other analyses. Given the 243 ng/ml value

of the extreme outlier fence in this analysis, we rounded this figure up to 250 ng/ml as a proposed Interim SLOD.

With regard to the lower screening limit for the dataset excluding the 4,200 ng/ml identification, a limit of 208 ng/ml is calculated. However, the higher 250 value we consider appropriate and present for a number of reasons. The first is the relatively small size of the dataset, indicated to us that it may be best to err on the side of caution using a higher value, as the presence of two mild outliers in such a small set raises the question of how representative the population studied herein is. Secondly, the current long in place threshold for Naproxen in the Mid-Atlantic States is 1000 ng/ml in plasma, indicating that other geographically related jurisdictions are using higher Naproxen thresholds. Also, the ratios of the pharmacologically based thresholds, as presented by Toutain et al. (2002b) and currently in place ARCI regulatory thresholds for therapeutic medications are in overall good agreement with an interim SLOD for Naproxen of 250 ng/ml. Further, in vitro pharmacodynamic studies demonstrate that 2,300 ng/ml Naproxen is required for the suppression of platelet aggregation in horses (Johnstone, 1983); support the concept of a no-effect SLOD of 250 ng/ml. Based on the Stevenson et al dose schedule and our pharmacokinetic model, it would take a similar therapeutic administration approximately 5 days to decline to this SLOD, on average.

Having identified the figure of 250 ng/ml for the Interim SLOD, the next question was how this Interim SLOD compares with other regulatory thresholds in place in North America. To perform this evaluation, we elected to compare the Association of Racing Commissioners International (ARCI) thresholds for controlled therapeutic medications with the 16 scientifically defined irrelevant plasma concentrations (IPCs) for the ARCI controlled therapeutic medications as presented by Toutain and Lassourd (2002a, b). In this work, Toutain and Lassourd use a pharmacokinetic analysis and a conservative safety factor to calculate their ineffective plasma concentrations (IPC) values for about 36 therapeutic medications, below which IPC value they considered that there was no possibility of pharmacological effect.

Reviewing this analysis, we note that the Toutain IPC for Naproxen is, at 26 ng/ml, about tenfold lower than our proposed Interim SLOD. However, when we compare the

ratios of the Toutain IPCs with the ARCI regulatory thresholds, as presented in Figure 4, we note that this 10-fold ratio for our interim Naproxen SLOD to the Toutain IPC is very close to the overall 9.6 ratio of Toutain IPCs to ARCI thresholds. Simply put, the ARCI regulatory thresholds are, on average, about 9.6-fold higher than the highly conservative Toutain and Lassourd IPC values, with the ratios ranging from less than one for Guaifenesin to 200-fold above the Toutain IPC for Phenylbutazone and 294-fold above the Toutain IPC for Omeprazole.

The next question that we addressed is the matter of the amount of environmental exposure that could give rise to these 161 ng/ml or lower plasma concentrations of Naproxen reported in the Charles Town samples. We therefore calculated the total daily exposure to Naproxen required to produce the plasma concentrations of Naproxen observed in these Charlestown samples. As shown in Figure 5, the smallest concentration identified, 6.3 ng/ml, required a Naproxen intake of little over 2 mg per day, in the order of 1/5,000 of the high-end 10 g daily dose required to produce a pharmacological effect in a horse. To produce the 161 ng/ml plasma concentration, the highest concentration assigned to an environmental origin, would require an intake of about 56 mg per day, or 1/178th of a clinical dose. In evaluating these data, we again draw attention to the post 80-hour data of Figure 1 from the Canadian research, where we note that essentially all of these Charles Town values are well below the post 80-hour Naproxen values that are most likely associated with reuptake of residual environmental Naproxen. The trace level plasma concentrations reported in the Charles Town samples are therefore quite consistent with residual traces in animals either treated with Naproxen or exposed to environmental Naproxen before shipping to Charles Town to race.

Figure 2.3.1.5 RMTC-to-Toutain-IPC Ratio

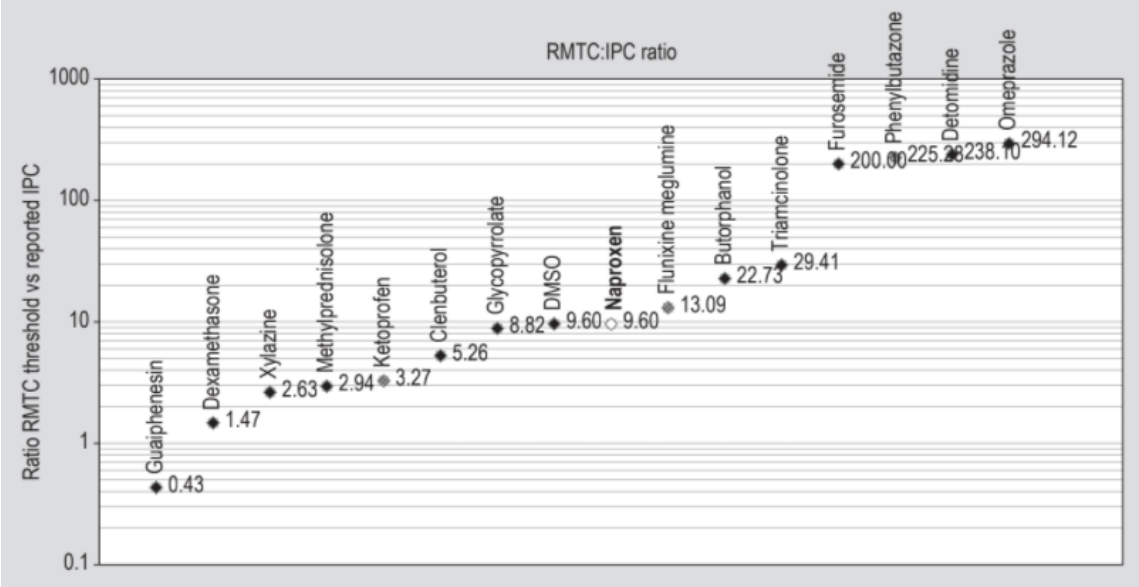

Figure 4. *RMTC/Toutain IPC ratios. This figure presents the ratios of the Toutain irrelevant plasma concentrations (IPCs) to the Racing Medication and Testing Consortium (RMTC) thresholds for the listed controlled therapeutic medications. In all but one case, Guaifenesin, the Toutain IPC is more conservative than the corresponding RMTC threshold. The ratios range from 1.5× more conservative for Dexamethasone to about 300× more conservative for Omeprazole. The proposed interim screening limit of detection of 250 ng/ml in plasma for Naproxen is close to 1/50th of the Toutain effective plasma concentration (EPC) and makes this interim screening limit of detection for Naproxen more conservative than those for Phenylbutazone and Flunixin and close agreement with the overall 9.6-fold ratio between the RMTC thresholds and the more conservative Toutain calculated IPC values.*

Figure 2.3.1.6 Estimated Plasma Concentration based on input of Naproxen

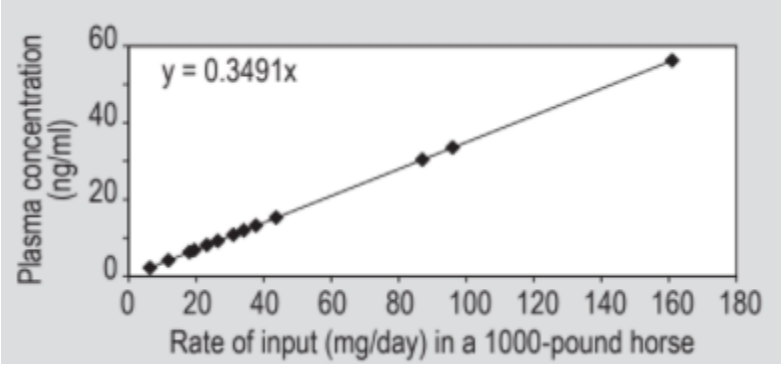

Figure 5. *Relationship of daily intake to plasma concentrations of Naproxen. The plotted concentrations were calculated assuming a steady-state concentration was measured from environmental exposure with the following equation: Css × CL = R0, where Css is the reported concentration, CL is the Clearance reported by Toutain (32 ml/ (kg h), and R0 is the calculated rate of input (in ng/(kg h)). Mg/day was calculated assuming a 1000 lb (454 kg) animal. Oral bioavailability (approximately 87%) (Cagnardi et al., 2011) is not included in this calculation.*

Review of the Naproxen identifications reported in 2016 and 2017 offers strong support for the hypothesis that these trace level identifications are associated with minimal and pharmacologically insignificant residues retained in horses coming into Charles Town from neighboring Mid-Atlantic States. Of the 11 identifications reported, only one was from a horse stabled at Charles Town. The other ten horses all shipped in from neighboring Mid-Atlantic States, 3 from Laurel Park, 1 from a VA training center and all of the other horses from farms in Virginia (4), and 2 from farms of unknown location. The horses yielding these trace level Naproxen identifications were therefore with one exception coming from neighboring Mid-Atlantic States with more tolerant Naproxen regulations, accounting for the finding of these trace levels concentrations of Naproxen in the plasma samples from these horses. The horsemen involved are, as a practical matter, likely completely unaware of the ability of Naproxen to persist in the environment and to therefore be present at trace levels in horses for an uncertain period after the last therapeutic administration.

These horses have therefore either inadvertently become exposed to small environmental amounts of Naproxen, the amounts of Naproxen being in the order of one 200^{th} or less of a single clinical dose of Naproxen or have been treated previously some

time ago such that the amount of Naproxen present is no longer pharmacologically relevant. Alternatively, since the time post the last Naproxen administration for a horse to 'clear' a test of the sensitivity of the current Charles Town testing is unknown, these identifications could also be the completely ineffective last traces of a therapeutic administration administered up to an unknown number of days before shipping to Charles Town. However, whatever the source of these identifications, they are completely unlikely to be associated with a pharmacological effect, and as such of no regulatory or forensic interest.

This phenomenon of inadvertent stall contamination driven exposure to equine therapeutic medications has been reported previously in the equine regulatory literature, starting with the 13th International Conference of Racing Analysts and Veterinarians (ICRAV) in Cambridge, UK. At this conference Norgren et al. (2000) showed Flunixin to give rise to stall contamination problems, which analysis was later confirmed when Popot showed Flunixin to be recycled in stalls via contaminated hay (Popot et al., 2011). Similarly, Russell and Maynard (2000) showed the presence of Isoxsuprine in a stall environment, i.e., food, bedding, wood scrapings, cobwebs, and the feeding manger of a horse. In this case, the Isoxsuprine administration had ended a full three weeks prior to the event in which the horse tested positive, with the stall sampling occurring at some significant further time after the race in question. Also, at this Cambridge conference, Williams et al. (2000) showed Ibuprofen to be to be detectable in the urine of horses whose feed had been prepared by contaminated human hands, and we have already referred to the Wennerlund et al. (2000) report on Naproxen.

The take home message is that equine treatment stalls inevitably become contaminated with the treatment medication. If the dose/amount of the medication is significant, if the substance is stable in the stall environment and absorbed orally, then the horse remaining in the treatment stall may be at risk of a medication identification for an undefined period of time. If such a treated horse is likely to be medication tested it is therefore wise to move the horse to a fresh stall, so that ongoing post-treatment background exposure of the horse to the treatment medication does not occur, and a realistic time for the horse to go 'testing negative' can be estimated. For example, in the present matter based on our pharmacokinetic model of Naproxen plasma levels, we estimate it would take a

standard therapeutic administration of Naproxen five or more days to on average fall below this proposed SLOD.

Other examples of inadvertent exposure of horses to human use substances are the now well-understood circumstances of exposure to trace amounts of morphine, generally in feedstuffs and appearing in equine urines as morphine glucuronides (Camargo et al., 2005). Other such substances are cocaine, appearing in post exposure urines as the major urinary metabolite benzoylecgonine (Camargo et al., 2006) and caffeine, appearing in post-race urine samples as parent caffeine and its various metabolites (Budjahara et al., 2007).

Additionally, given the circumstances of this current Charles Town situation, where neighboring states had higher regulatory thresholds for Naproxen, one practical regulatory approach is an appropriate SLOD so that medications identifications are not reported based on pharmacologically insignificant trace levels of the medication in question, as has been the case in most of the inadvertent exposure circumstances described above.

Conclusions

As well as being of no pharmacological significance, it is also equally clear that the identification and reporting of these low concentration Naproxen identifications has been a direct result of the application of highly sensitive plasma testing for Naproxen. As such, this sequence of sporadic low concentration identifications commenced essentially immediately after testing transferred to a laboratory using highly sensitive testing for Naproxen. The immediate solution to this problem, therefore, is to introduce an interim SLOD for Naproxen in the order 250 ng/ml Naproxen (or 1.09 uM), as we have outlined in this communication.

Conflict of interest

The authors recognize no conflicts of interest in the publication of this paper.

Acknowledgements

Published as paper #500 from T Tobin and the Equine Pharmacology, Therapeutics and Toxicology Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky. The information

reported in this paper is part of a project of the Kentucky Agricultural Experiment Station (KAES publication # 15-14-036) and is published with the approval of the Director. This research was supported by grants from the USDA Agriculture Research Service Specific Cooperative Agreement #58-6401-2-0025 for Forage-Animal Production Research, the Kentucky Department of Agriculture and the Kentucky Thoroughbred Association Foundation and by support for the Kentucky Agricultural Experiment Station as provided by the National Institute of Food and Agriculture (NIFA) and the Commonwealth of Kentucky. Other support that has made this work possible includes research support from The National Horsemen's Benevolent and Protective Association and the Alabama; Arizona; Arkansas; Ontario, Canada; Charles Town, WV; Florida; Indiana, Iowa; Kentucky; Louisiana; Michigan; Minnesota, Nebraska, Ohio, Oklahoma, Ontario

Canada, Oregon, Pennsylvania, Tampa Bay Downs, Florida; Texas; Washington State; and West Virginia Horsemen's Benevolent and Protective Associations, the Equine Health and Welfare Alliance, Incorporated, Versailles, Kentucky and Mrs. John Hay Whitney.

References

Baldwin, A.C., Stevenson, S.W. and Dudley, G.A., 2001. Nonsteroidal anti-inflammatory therapy after eccentric exercise in healthy older individuals. Journal of Gerontology Series A 56: M510-M513.

Barker, S.A., 2008. Drug contamination of the equine racetrack environment: a preliminary examination. Journal of Veterinary Pharmacology and Therapeutics 31: 466-471.

Brewer, K., Shults, T.F., Machin, J., Kudrimoti, S., Eisenberg, R.L., Hartman, P., Wang, C., Fenger, C., Beaumier, P. and Tobin, T., 2016. A cluster of trace-concentration methamphetamine identifications in racehorses associated with a methamphetamine-contaminated horse trailer: a report and analysis. Canadian Veterinary Journal 57: 860.

British Horseracing Authority, 2015. Results of enquiries. (J. Doyle, Christmas Spirit, E. Williams, J. Scott, Various Trainers, A. Bailey, W. Treacy) heard by disciplinary panel on Thursday 24 September. Available at: https://tinyurl.com/yyb7zrx5

Budhraja, A., Camargo, F.C., Hughes, C., Lehner, A.F., Stirling, K., Brennan, N., Dowling, M., and Tobin, T., 2007. Caffeine and theobromine identifications in post-race urines: threshold levels and regulatory significance of such identifications. AAEP Proceedings 53: 87-92.

Cagnardi, P., Gallo, M., Zonca, A., Carli, S. and Villa, R., 2011.Pharmacokinetics and effects of alkalization during oral and intravenous administration of naproxen in horses. Journal of Equine Veterinary Science 31: 456-462.

Camargo, F.C., Hughes, C., Lehner, A.F. and Tobin, T., 2006. Trace level benzoylecgonine identifications in post-race urines: probable sources and the regulatory significance of such identifications. AAEP Proceedings 52: 331-336.

Camargo, F.C., Lehner, A.F., Karpiesiuk, W., Stirling, K., Kavanagh, P.V., Brennan, N., Dowling, M. and Tobin, T., 2005. Review of environmental morphine identifications: worldwide occurrences and responses of authorities. AAEP Proceedings 51: 58-64.

Davies, N.M. and Anderson, K.E., 1997. Clinical pharmacokinetics of naproxen. Clinical Pharmacokinetics 32: 268-293.

Dudley, G.A., Czerkawski, J., Meinrod, A., Gillis, G., Baldwin, A. and Scarpone, M., 1997. Efficacy of naproxen sodium for exercise-induced dysfunction muscle injury and soreness. Clinical Journal of Sport Medicine 7: 3-10.

Johnstone, I.B., 1983. Comparative effects of phenylbutazone, naproxen and flunixin meglumine on equine platelet aggregation and platelet factor 3 availability in vitro. Canadian Journal of Comparative Medicine 47: 172-179.

Lanier, A.B., 2003. Use of nonsteroidal anti-inflammatory drugs following exercise induced muscle injury. Sports Medicine 33:177-186.

Lees, P. and Higgins, A.J., 1985. Clinical pharmacology and therapeutic uses of non-steroidal anti-inflammatory drugs in the horse. Equine Veterinary Journal 17: 83-96.

Machin, J., Shults, T., Fenger, C. and Tobin, T., 2017. Inadvertent environmental transfer of dextromethorphan from groom to racehorse results in establishment of a pharmacologically relevant cutoff. Toxtalk 41: 20-23.

Norgren, A., Ingvast-Larsson, C., Kallings, P., Fredriksson, F. and Bondesson, U., 2000.

Contamination and urinary excretion of flunixin after repeated administration in the horse. In: Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R&W, Cambridge, UK, pp. 377-380.

Popot, M.A., Garcia, P. and Bonnaire, Y., 2011. Doping control in horses: housing conditions and oral recycling of flunixin by ingestion of contaminated straw. Journal of Veterinary Pharmacology and Therapeutics 34: 612-614.

Russell, C.S. and Maynard, S., 2000. Environmental contamination with isoxsuprine. In: Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R&W, Cambridge, UK, pp. 381-383.

Snyder, S.A., 2008. Occurrence, treatment, and toxicological relevance of EDCs and pharmaceuticals in water. Ozone: Science and Engineering 30: 65-69.

Soma, L.R., Uboh, C.E., Rudy, J.A. and Perkowski, S.Z., 1995. Plasma and synovial fluid kinetics, disposition and urinary excretion of naproxen in horses. American Journal of Veterinary Research 56: 1075-1080.

Stevenson, A.J., Weber, M.P., Hopkins, D. and Kacew, S., 1997. Anti- inflammatory drugs. Analytical methodology for detection and confirmation of drugs in equine body fluids. Vol. III. Canadian Equine Drug Evaluation Program – Canadian Pari-Mutuel Agency, Agriculture and Agri-Food Canada, Ottawa, ON, Canada, pp. 194-207.

Tobin, T., 1981. Drugs and the performance horse. Charles C. Thomas, Springfield, IL, USA.

Tobin, T., Dirikolu, L., Brewer, K. and Hughes, C.G., 2013. A clinician's guide to factors affecting withdrawal times for equine therapeutic medications. Veterinary Journal 198: 313-321.

Toutain, P.L. and Lassourd, V., 2002a. Pharmacokinetic/pharmacodynamic approach to assess irrelevant plasma or urine drug concentrations in postcompetition samples for drug control in the horse. Equine Veterinary Journal 34: 242-249.

Toutain, P.L. and Lassourd, V., 2002b. Pharmacokinetic/pharmacodynamics assessment of irrelevant drug concentrations in horse plasma or urine for a selection of drugs. In: Proceedings of the 14th International Conference of Racing Analysts and Veterinarians.Orlando, FL, USA.

Wennerlund, I., Ingvast-Larsson, C., Kallings, P., Fredriksson, E. and Bondesson, U., 2000. Pharmacokinetics and urinary excretion of naproxen after repeated oral administration. In: Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R&W, Cambridge, UK, pp. 195-200.

Williams, R.B., Woodward, K., Hines, S. and Teale, P., 2000. Urinary detection of ibuprofen: feed contamination, topical application and oral administration. In: Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R&W, Cambridge, UK, pp. 372-376.

World Anti-Doping Agency (WADA), 2017. Decision limits for the confirmatory quantification of threshold substances. WADA Technical Document – TD2017DL. Available at: https://tinyurl.com/y6y5yna7.

World Anti-Doping Agency (WADA), 2019. What is prohibited. Available at: https://tinyurl.com/y7fyyqkr.

Zuccato, E., Calamari, D., Natangelo, M. and Fanelli, R., 2000. Presence of therapeutic drugs in the environment. The Lancet 355: 1789-1790.

CHAPTER 3. CONCLUSION

3.1 Biologically relevant cut-off determination

Perhaps the model example of determining a physiologically-relevant cut-off is the system proposed by Toutain – that is, the determination of Effective Plasma Concentrations [EPCs] and their related regulatory Irrelevant Plasma Concentrations [IPCs]. The calculation of an EPC is straightforward – divide the standard dose (per dosing interval) by the plasma clearance (per dosing interval). Toutain, in his original article published in the International Conference of Racing Analysts and Veterinarians calculated the EPC for thirty-six compounds. Unfortunately, many of the compounds listed by the ARCI are not previously calculated, but when the pharmacokinetic data is available for a compound, such a calculation is rudimentary. For example, for the non-steroidal anti-inflammatory drug ketoprofen, the dose (in mg/kg) is 2.200, the dosing interval (in hours) is 24, and the clearance (in mL/kg/h) is 300. Taking these values, then, we can determine an EPC of 306 ng/mL in plasma, as shown below.

$$EPC = \frac{2.2\ mg/kg\ per\ 24\ hours}{300\ ml/kg/h * 24\ hours} = \frac{2.2 mg/kg}{7200\ ml/kg} = \frac{3.06*10^{-4}\ mg}{mL} = 306 \text{ ng/mL}$$

An IPC then, is determined by dividing the EPC by some safety factor that should be determined by a regulatory body. For Toutain's initial paper, a safety factor of five hundred (500) was used in his example calculations, though he notes that it is up to regulators to determine the factor that is most appropriate for a given therapeutic compound. As was shown in the naproxen threshold paper, the RMTC's current regulations vary widely in what is effectively the safety factor for their drugs (ranging from a safety factor of roughly 1200 (guaifenesin) to a safety factor of approximately 1.7 (omeprazole). However, generally the safety factors tended to be around the value of fifty (50), indicating that when other considerations are excluded, this is a reasonable level to set the EPC: IPC safety factor ratio.

3.2 Determining an appropriate safety factor

What outside factors are likely a source of concern for regulators? Ultimately, there are two major categories of factors to take into consideration: the potential impact on the fairness of a race, and the protection of the animal's health. Those compounds that may impart a competitive advantage to an animal may be more stringently regulated than those that offer little to no benefit on race day, and those compounds which are likely to have a minimal impact on a race are likely to be allowed a closer safety factor. For example, the relatively high value allowed threshold for omeprazole (10 ng/mL in plasma) compared to its calculated EPC (17 ng/mL) may partially be attributed to the fact that there are no known performance enhancing effects of the compound on aerobic performance in performance horses (Kollias-Baker, et al 2001).

3.3 Thresholds vs Cut-offs

Pharmacologically therapeutic compounds are perhaps the most straightforward to regulate in such a manner and testing limits on these are referred to as 'thresholds.' However, other compounds may be present due to environmental, dietary, or naturally occurring within the animal that may be of regulatory relevance to the industry. These compounds are deemed Environmental, Dietary, and Endogenous [EDE], and may require a more subtle approach, as the pharmacokinetics of many of these compounds are less likely to be studied or their pharmacology may prove irrelevant to the establishment of practical regulatory limits (called 'cut-offs' for these types of compounds). The naproxen paper discussed in Chapter 2.3 demonstrates this as well. Sporadic 'positives' called by regulatory bodies often indicate a source of unknown or uncontrolled contamination by horsepersons, such as was likely with the naproxen 'positives' called out of Charles Town Races. Using statistical outlier methods, screening limits may be established for such compounds to determine what is likely the result of a therapeutic dose compared to an environmental exposure. These data may take time to gather, as it relies upon sporadic exposure events to make a horse test 'positive', but they offer good insight into a practical cut-off. Whenever this type of data is not accessible, environmental contamination studies

may be necessary (such as occurred at Charles Town Racing), or for the case of endogenous substances, such as testosterone, population models must be properly constructed.

3.4 The "Zero-Tolerance" dilemma

Ultimately, a zero-tolerance approach is not only impossible to test for, but impractical to enforce as modern detection methods push the limits of analytical sciences. One example of easy visualization can be thought of as follows. Using reported data for naproxen, as an example. A five-gram oral dose will reach its terminal recorded half-life of 80 hours after approximately 72 hours, with about 50 milligrams of the compound remaining in the animal. With a molecular mass of 230.259 g/mol, that is approximately 1.31E+20 molecules remaining in the horse. Assuming the reported terminal half-life remains constant from 72 hours onwards, it would take approximately 222.75 additional days for the compound to fully clear the animal's system (225.75 total days). As the RMTC does not list this common therapeutic as having a threshold, a horseperson then would be expected to withdraw their animals for more than half of a year after the administration of a single well-recommended therapeutic NSAID. Obviously, this level of therapeutics restriction goes too far if taken to this extreme.

Figure 2.3.1.1 Time to ‘Zero Tolerance’ Acceptable Levels of Naproxen after Dosing

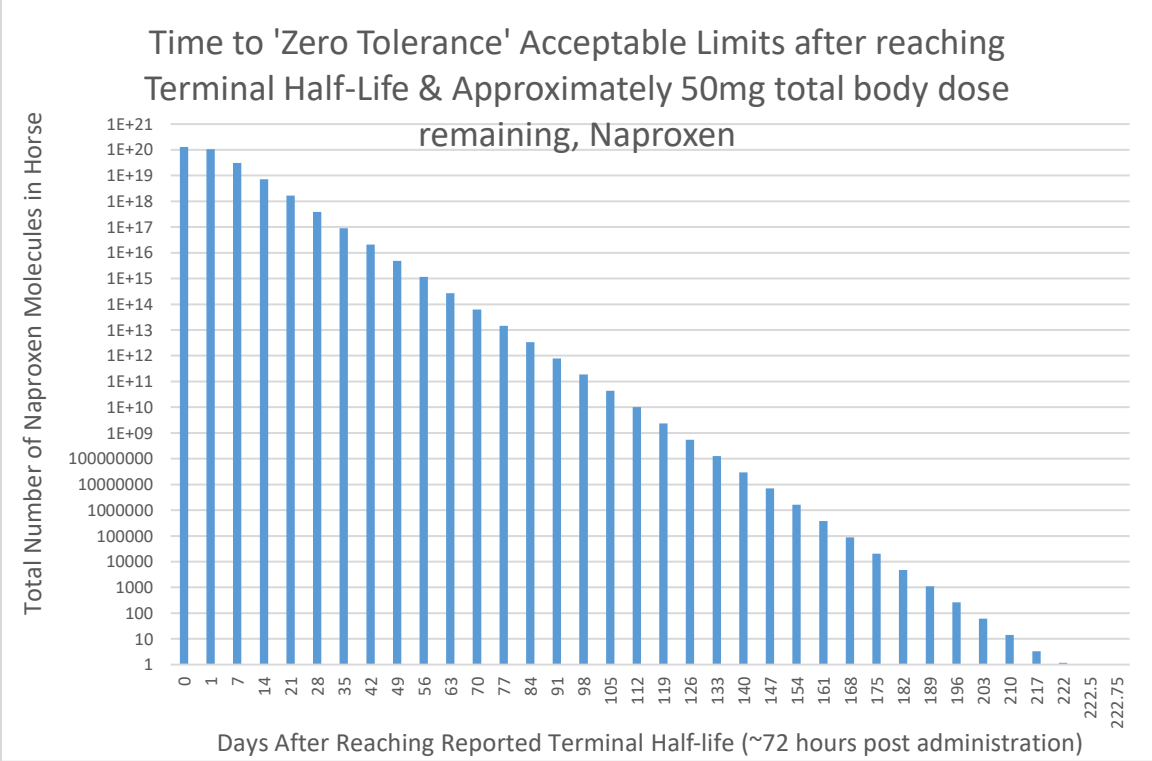

Figure shows the expected molecule count of naproxen over time upon reaching a total body dose of 50mg, based upon measured and reported terminal half-life.

Some compounds, however, remain within the realm of ‘zero detection’ limits having use. For instance, synthetic anabolic steroid may be of interest in that they negatively impact the health of the animal while giving it an advantage when racing on the track even when it is below detectable limits by gaining a large amount of muscle mass during training. For compounds such as these and other long-term performance-enhancing drugs, a zero-tolerance policy may be appropriate. However, it is worth noting that such policies should always be the exception rather than the rule due to the sensitivity of modern testing (which will only continue to become more sensitive) and it may be necessary in the future for thresholds for such substances to be implemented.

3.5 No Mediating Bodies

One of the primary challenges facing regulation in the horseracing industry is the level of translational research that must be done to bring both veterinarians, equine

scientists, horsepersons, and regulators together. Forensic equestrian scientists may be experts in the field of isolating and identifying pharmacological compounds in the media presented to them, but they are rarely well-equipped to also understand the underlying physiological effects for what a specific compounds concentration represents. Likewise, regulators may have great understanding of what rules exist and how they are being applied within their jurisdiction, but they are not necessarily experts at understanding neither the analytical science behind the detection event itself, nor the underlying physiological relevance of said call. What perhaps would best be proposed is a mediating body through which regulators and analysts may communicate. Such a body could be staffed by veterinary scientists that are well trained in both the regulatory desires as well as the science, both analytical and veterinary. Fortunately, an example of just such a body is readily available to study, as the human drug-testing standards within the United States go through such a body, as established by the Substance Abuse and Mental Health Services Administration [SAMHSA] and their Medical Review Officers [MROs].

3.5.1 How it works in humans – SAMHSA

The Substance Abuse and Mental Health Services Administration was formed in 1992 as part of a reorganization of governmental bodies due to the abolition of the Alcohol, Drug Abuse, and Mental Health Administration [ADAMHA]. It was directed to effectively target drug abuse and mental health services to the people most in need, with a focus on translational research in those areas. Initially, SAMHSA was directed to test for five commonly abused drugs – amphetamines, cocaine, marijuana, opiates, and phencyclidine [PCP]. Today, they test for a total of nine (9) compounds for governmental organizations as well as for the public – Methamphetamine (d-Methamphetamine/d-Amphetamine), Marijuana (THCA, 11-nor-Δ^9-THC-COOH), Cocaine (Benzoylecgonine), Opiates (Codeine/Morphine), PCP (Phencyclidine), Heroin (6-Acetylmorphine, 6-AM), Ecstasy (MDMA/MDA), Vicodin/Dilaudid (Hydrocodone/Hydromorphone), and OxyContin (Oxycodone/Oxymorphone). Each of these substances has a screening cutoff, as shown in the following table:

Table 3.5.1.1 SAMHSA Cut-offs for Regulated Substances in Humans

Common Name	Parent Drug/Metabolite	Cut-off (ng/mL)
Meth	d-Methamphetamine/ d-Amphetamine	500
Marijuana	THCA (11-nor-Δ^9-THC-COOH)	50
Cocaine	Benzoylecgonine	150
Opiates	Codeine/Morphine	2000
PCP	Phencyclidine	25
Heroin	6-Acetylmorphine (6-AM)	10
Ecstasy	MDMA/MDA	500
Vicodin/Dilaudid	Hydrocodone/ Hydromorphone	300
OxyContin	Oxycodone/ Oxymorphone	100

Several of these screens require secondary confirmation of specific analytes to be considered a positive test. In all cases that a cut-off/threshold is exceeded, however, immediate action is not taken, but the data is forwarded to a Medical Review Officer, a physician (MD) who has trained in understanding substance abuse, the methods for testing, and analysis of the data from such tests. At this point, the medical review officer will request a valid prescription or physician's verification of medical treatment in support of the positive drug test. Failure to provide such results are likely to result in the reporting of the test as a 'positive' to the relevant authorities (whether governmental or otherwise).

However, of import is that a well-trained Medical Review Officer has some level of discretion for which to proceed. This, coupled with his understanding of the testing facilities and regulations, enables him to have a good understanding of the situation in cases of 'borderline' positives, sparing the time, energy, and money of both the subject of the test and those ordering such a test. While for certain areas this discretion may be greatly

reduced (for instance, in testing of prison inmates), in others it may be more broadly applied (in the case of private employers, for example).

One of the other points to note for the SAMHSA model is that the values for these cut-offs is noticeably higher than those for the equine industry. This is reasonable when one considers that humans are often subjected to a much less controlled environment than a racehorse (public transportation, shared housing, etc.), but the degree to which compounds are reported as 'positive' in the equine industry is orders of magnitude lower than in human testing, as demonstrated by Brewer, et al in their paper covering the several 'positive' methamphetamine call in Toronto for concentrations (200, 56, and 340 picograms-per-milliliter of urine). That paper supports a practical interim screening limit (threshold) of 15 nanograms-per-milliliter, due to the chance of environmental contamination (as was shown to be the cause by investigators in that case). This is still a factor of thirty lower than testing for humans, but a reasonable approach considering that equine exposure to contaminated environments is likely minimal compared to the risk humans face of coincidental exposure.

3.6 Summation of Research

The design and implementation of appropriate pharmacokinetic studies, both for general administration of therapeutic compounds and for those which may be environmental contaminants is critical to the development of appropriate regulations regarding pharmacologically relevant compounds in the performance sports horse. Critical to these types of studies are the novel syntheses of references standards as well as stably isotopically labelled internal reference standards and the availability of such compounds to the public. The work undertaken for this thesis has focused on all of these aspects.

As a summation of the specific work, those cited as primary authorships include the following syntheses: xylazine-d6, barbarin, and barbarin-d5. Those studies which focused on trace therapeutic medication overages: mepivacaine, methylprednisolone, and

betamethasone. Finally, a paper on the environmental contamination as a source of naproxen positives was also included.

These studies have focused on substances that have confounded regulators for some time, due to both improvements in sensitivity in testing as well as the sporadic nature of environmentally sourced substances. However, more work has been completed since that time as well and following are collaborations that have come forward either as a direct result, or alongside this thesis' primary course of study.

The development of a novel synthesis for xylazine-d6 led to a collaborative study, headed by Dr. E. Macomber making a recommendation of a threshold of 200 pg/mL in plasma for a withdrawal time of 48 hours. Prior to such pharmacokinetic research, the regulatory threshold for this withdrawal guideline was 10 pg/mL. Today, the RMTC recommends the suggested 200 pg/mL threshold, showing the value of such studies in guiding the regulatory bodies. This research was not only communicated with the RMTC, but also submitted to the Veterinary Journal for publication (Macomber, et al 2017).

Similarly, our work with betamethasone also helped with another collaborative study focusing on betamethasone's isomer: dexamethasone. This study, led by Dr S. McClure indicated that a daily dose of intravenous dexamethasone demonstrates no accumulation, and so withdrawal guidelines for horsepersons should not change based on multiple dosings. While that may seem insignificant, the animals that will continue to receive appropriate medical treatment without their owners fearing for an accidental overage is of great importance. This study also led to an interesting environmental contamination study reported in the same paper, in which it was shown that urine containing dexamethasone (the form by which it is excreted in the horse) in the environment is concentrated enough to be detectable in the blood untreated horses that are exposed. This study was published in Comparative Exercise Physiology (McClure, et al 2021).

Several articles have also been published in non-scientific trade journals based upon our research. These include a review of the Charles Town Ship-In Stalls and the contamination found within them. This study found that seventeen of twenty-one stalls tested at Charles Town Racing tested positive for environmental contamination of pharmacologically relevant compounds. This included twenty-five total substances, some of therapeutic value for both humans and horses as well as human recreational substances. Personal communications also indicated that all stalls that housed animals tested positive for detectable levels of naproxen, though only those above a specific threshold were reported as positive. This underscores the importance of developing thresholds for most substances, as it is an inevitability that animals have the potential to be exposed to said compounds in their environment, regardless of the hygienic practices of their owners and trainers (Fenger, et al. 2017).

Similar to Charles Town's stall contamination, a series of tramadol positives was reported on, which is believed to be the result of trace transfers between horsepersons and their animals. Tramadol, an opioid pain medication, is a common prescription for elderly horsepersons as they often continue to lead physically demanding lives. Often prescribed in the form of a transdermal patch that the patient wears, contamination on the fingertips after application may result in positive tests when they go on to use equipment such as tongue ties. It is also possible that human urine could be one of the transmission methods for tramadol, as it is common practice to relieve themselves in the stalls of the animals when finding a restroom may prove inconvenient (Brewer, et al 2018).

The first publication that the author was a contributor to, was in fact a case of trace contamination resulting a 'positive' call. Environmental exposure does not only pertain to therapeutic and naturally occurring compounds. One such example is with the illicit methamphetamine. Methamphetamine is not a therapeutic that has any legitimate use in equestrian sport, however a trainer and owner at Ajax Downs, Toronto, Ontario had 'positives' called against them when their horses competed. Of the four horses that ended their races in the money, three of them tested positive for methamphetamine at remarkably low urinary levels, approximately between fifty and three-hundred-fifty picograms-per-

milliliter. For comparison, in the United States, humans are generally tested by the standards of the Substance Abuse and Mental Health Services Administration [SAMHSA], which denotes that amphetamine, and its derivatives have a testing threshold of 500 nanograms-per-milliliter in urine, a factor of approximately five orders of magnitude more than what was detected in the racing horses.

However, the trainer was held accountable. Having appealed the decision, an investigation was undertaken. The three animals that tested positive all were shipped from Atlanta to Toronto in the same trailer while the one that had tested 'clean' was in a separate trailer. Examination of the trailer showed trace levels of methamphetamine contamination – likely due to it being used as a mobile meth lab before being purchased by the horse owner. Charges were eventually dropped by the commission as it appeared that the trainer and owner had no way of knowing about the contamination. A suggested fifteen nanogram-per-milliliter threshold was suggested based on this data, still very conservative compared to those used in human testing (Brewer, et al. 2016).

Finally, perhaps the most interesting single line of research, the novel syntheses of barbarin and its deuterated variant, culminated in the positive identification of a common weed (*Barbarea vulgaris*) as a potential source of aminorex positives appearing in the urine of horses. This research, led by Dr. G. Maylin, showed that horses will readily eat this plant, containing glucobarbarin – the glucosinolate precursor of barbarin – when it is mixed with their food, and upon its consumption their urine will in fact have detectable levels of aminorex present. Furthermore, it is likely that several other species that have been identified as producers of barbarin may likely also cause potential aminorex 'positives' should they be consumed by the animal (Maylin, et al 2019).

3.7 Conclusion

Ultimately, it is studies such as these that will allow for the better regulation and protection of equine athletes. However, for such studies to have any impact, regulators must understand the need for thresholds in protecting the health of the animals as well as the sport. The first step of this consists of identifying pharmacologically relevant

substances in the environment. Most often, this occurs whenever a series of low-level 'positives' appear within the regulatory environment in a relatively short period of time. This may be due to changing of internal regulatory methods (as was the case in West Virginia whenever they changed analytical laboratories), seasonal crop/plant growth exposing the animals to environmental contamination (as may be the case for some aminorex positives linked to *B. vulgaris*), or other sources. However, it can generally be understood that if wrongdoing is being undertaken by horsepersons, a 'positive' should act as a warning flag and future positives should not continue to appear sporadically as they often do with environmental contaminants.

Upon recognition of such a substance, the analytical methodologies for its characterization and detection need to be developed. While these methodologies are often straightforward, the availability of reference standards and internal standards is often quite scarce for many pharmacologically relevant compounds and their metabolites. As such, the novel syntheses of both the parent compound, the metabolite, and stably-isotopically-labelled versions of these substances is of paramount importance in maintaining the integrity of the testing environment. Such syntheses have been undertaken for the production of xylazine-d6, barbarin, and barbarin-d5, all of which are now available as reference standards to the regulatory and research communities.

Once suitable reference standards are available, pharmacokinetic studies are of utmost importance in describing the ability of a compound to remain within the body of the horse at detectable levels. As analytical techniques improve, these levels will be pushed to smaller and smaller quantities, with regulations commonly on the pg/mL scale, with some substances having regulatory 'positives' at the fg/mL scale. At these concentrations in the body, it is likely that the elimination is much slower than reported in previous studies, as it reaches a different rate of metabolism. This has been shown to be the case for several compounds, notably mepivacaine, methylprednisolone, and betamethasone having substantially longer measurable terminal half-lives than previously reported.

The importance of accurate terminal half-lives is difficult to understate with regards to equine therapeutic regulation. That is because the current system within the United States is designed around 'withdrawal guidelines' that offer a time by which a horse should no longer test above the established testing threshold. Unfortunately, many of these thresholds were determined rather arbitrarily low (or at least with little to no scientific evidence publicly available), and the methodologies used to determine the relevant withdrawal guidelines often failed to capture the terminal half-life associated with such small concentrations.

Not only must long terminal half-lives be properly identified and published, but the possibility for urinary-oral reabsorption should be considered for environmentally stable or highly bioavailable compounds. The potential for such compounds to contaminate the area around the animal via urine and then be reabsorbed has been shown to a potentially confounding variable which should be addressed by racing regulators whenever possible. Naproxen is one such example, as shown.

One of the most practical ways to help regulators prepare their rules for the future is to focus on thresholds that are based on physiologically relevant concentrations, rather than arbitrary detection limits and withdrawal timelines. To do this is straightforward, only requiring the calculation of an Effective Plasma Concentration and then determining an appropriate safety factor for the compound by which to produce an Irrelevant Plasma Concentration to act as a threshold for said substance. For most compounds, a safety factor of fifty seems appropriate, though individual substances should be considered against their own class of pharmaceuticals, their potential to influence the fairness of a race, and their need for the health of the horse.

Finally, regulators may be advised to consider the development of a mediating body between themselves and the analytical/forensic laboratories that test these samples. These mediating bodies would be comprised of specialists that would understand the intersections of analytical chemistry, veterinary sciences, and the regulatory structure of the horseracing commissions. They would understand the field to ascertain what concentrations are likely

a problem and those that are likely irrelevant, notice any trends developing with certain medication overages, and act to guide regulators to efficient and useful rulings should a 'positive' be identified. Likewise, these review officers could act within a specific jurisdiction and so understand the geographical trends that may develop, avoiding misunderstandings whenever testing facilities are changed with differing internal thresholds, and more.

Ultimately, the horse racing industry relies upon the testing facility and their regulations to maintain a level playing field for all involved, assure the public and bettors of the horsepersons' honesty, and most importantly, protect the health and wellness of the equine athletes. However, without proper regulatory adjustments, it will only be a matter of time before another high-profile case, as has happened with Medina Spirit, brings the industry to the mass media's attention, and perhaps permanently mar the reputation of the sport.

APPENDIX

Published papers not included in full above

Below are summaries of related papers that the author is included contributed to, but not as the primary author. They follow the following format:

Article title

Abstract

Experimental (excluded in review articles)

Conclusion

Citation

Regulatory thresholds for xylazine; review and analysis based on recent pharmacokinetic data

Abstract: Xylazine is an α2-adrenergic receptor agonist and a short acting sedative/analgesic widely used in equine practice since its original approval by the FDA in 1988. Closely related agents include Detomidine, Medetomidine, Romifidene, Amitraz and Guanabenz. Xylazine is the shortest acting member of this group and is a Racing Medication and Testing Consortium (RMTC) "Controlled Therapeutic Medication" (version 2.2); the RMTC interim threshold for xylazine was set at 10 pg/ml plasma with a 48-hour withdrawal and no defined dose. Application of this regulatory threshold in Washington State led rapidly to an apparent therapeutic overage of about 75pg/ml following a 200 mg dose IV at 54 hours prior to post. Based on Toutain's reported Irrelevant Plasma Concentration (IPC) for xylazine (2013) and the very short duration of action of xylazine, an interim 300 pg/ml regulatory threshold for xylazine was proposed. Pharmacokinetic data for xylazine up to 12 hours post administration shows that the terminal elimination of xylazine slows markedly from 6 hours post-administration, leading to a flat terminal half-life. The result of this flat terminal elimination curve for xylazine is that it can be detected in plasma for hours to days beyond any pharmacologic effect of the drug. Based on these considerations, the regulatory threshold for xylazine in Washington State was adjusted upwards to 200 pg/ml on an interim basis. Following this adjustment, review of reported plasma concentrations of xylazine in Washington State post-race samples suggests that this 200pg/ml in plasma adjusted interim regulatory threshold is likely a more appropriate and clinically relevant 48-hour post administration regulatory threshold for xylazine.

Introduction: Xylazine is one of the oldest and most commonly used analgesic and sedatives in the horse industry. It's rapid onset and brief duration make it ideal for use alone or with the coadministration with other agents. It has been shown to have a peak effect within ten minutes of intravenous administration, with return to clinical normalcy after only 90 minutes. Unlike detomidine, the Variable Interval Responding technique (used to determine subtle drug effects after the primary effect has worn off) has been shown to have no measurable change in response from normal after twenty-four hours. Other pharmacological effects of α2-adrenergic receptor agonists (decrease in packed cell

volume, protein, and increase in glucose) have all been shown to return to normal faster for xylazine (within approximately sixty minutes). All of these indicate that xylazine is and should remain one of the primary analgesic/sedatives used in the equine industry.

At the time of writing, the recommended threshold by the RMTC was 10 pg/mL, with a withdrawal time of 48 hours. No dose was defined at that time.

Positive cases that were for reasonably low doses (200mg via IV bolus; the recommended dose by the manufacturer is approximately 1mg/kg, roughly 450mg in an animal) resulted in racetrack positives at times greater than the 48-hour withdrawal timeline. The plasma concentrations reported were seven to nine times higher (a split sample returned a concentration of 0.093 ng/mL, or 93pg/mL). With that in mind, a review of the literature was determined to be necessary for a more appropriate screening limit.

Due to data published by Noble, along with Toutain's published Irrelevant Plasma Concentration data, an interim threshold of 200pg/mL was recommended and adopted by the Washington State Horse Racing Commission, and eventually by the Racing Medication & Testing Consortium as well.

Conclusion: Review of the pharmacokinetic data for xylazine suggested a terminal plasma half-life considerably longer than previously understood, and no publication was provided or referenced in the previous threshold of 10pg/mL. Field experience to date seems to indicate that the newly adopted 200pg/mL threshold is more appropriate, both pharmacologically and forensically.

Citation: Macomber, E., Noble, G., Machin, J., Morales, B., O'Connell, M., Stirling, K., … & Tobin, T. (2017) Regulatory Thresholds for xylazine; a review and analysis based on recent pharmacokinetic data. Submitted to the Veterinary Journal.

Dexamethasone serum concentrations after intravenous administration in horses during race training

Abstract: Dexamethasone (DXM) sodium phosphate is a widely used corticosteroid for inflammatory conditions in horses, regulated in racing jurisdictions in the United States by a 0.005 ng/mL serum/plasma threshold. This study seeks to describe serum concentrations of DXM at 48 and 72 hours after intravenous administration of 20 mg DXM sodium phosphate over 1 to 5 days, and to identify a possible source of DXM overages. Seventy-four horses (39 Thoroughbreds, 13 Standardbreds, 22 Quarter Horses) in active race training received 20 mg DXM sodium phosphate. Serum was collected before injection, at 48 and 72 hours post last injection, and analyzed by LC/MS-MS (Limit of Quantitation (LOQ) = 2.5 pg/mL). No differences were identified by ANOVA ($p \leq 0.05$) for racing breeds, age, gender or the number of days of DXM sodium phosphate administration, so data were pooled for each time point. The DXM serum concentration at 48 hours (mean ± standard deviation, SD, range) was 2.18 ± 1.56 pg/mL. (<2.5 to 40 pg/mL). Summary statistics could not be derived for 72-hour DXM serum concentration owing to censored data but ranged from <2.5 to 95.8 pg/mL. There was one extreme outlier (Tukey) at 48 hours, and two extreme outliers at 72 hours. A separate study was conducted using sedentary experimental horses to determine the likelihood that positive DXM samples could result from environmental transfer. Urine was collected from a mare 2 to 3 hours post administration of 20 mg DXM. Hay with 100mL of DXM (17 ng/mL) containing urine was offered to each of six experimental horses and blood was collected at 0, 4, 8, 12, 16, 20 and 24 hours. All six horses had plasma DXM concentrations above the LOD and five of six had plasma DXM concentrations above the LOQ for at least one sample time.

Experimental: Privately owned animals (Thoroughbred, Standardbred, and Quarter Horse racehorses) were used throughout the study. Training adhered to standard regimens consistent with the type of racing specific to the racing discipline. Inclusion criteria included a full clinical examination by a veterinarian. Exclusion criteria were injections with Betamethasone or DXM within 7 days, or any other medication within 24

hours prior to blood collection. Blood samples were drawn into 10mL serum separator vacuum tubes immediately preceding the first dose of DXM and at 48 and 72 hours after the last dose of DXM. These were allowed to clot, refrigerated, and centrifuged within 4 hours before being stored at -70 Centigrade. Serum Analysis was performed via LC-MS/MS using Selective Reaction Monitoring. Statistical/Pharmacological analyses were used, including a Robust Regression on Order and General Linear Model statistical methodologies.

The contamination experiment was performed by dosing Lucerne hay with urine that was collected from a mare previously administered 0.05mg/kg DXM IV. Blood samples from these six sedentary horses were then collected up to 24 hours after the exposure to the hay, and samples were analyzed by a more sensitive LCMS methodology with a Limit of Detection of 0.5pg/mL.

Conclusion: Results indicate that a daily dose of 20mg IV of DXM demonstrates no accumulation, and so withdrawal guidelines should not change for an animal requiring multiple dosings. The threshold of 5pg/mL at 72 hours shows an experimental positive test risk of approximately 2.7%. Similarly, environmental contamination from urine was shown to result in detectable levels of DXM in the blood of the horse.

Citation: McClure, S., Fenger, C., Kersh, K., Brown, B., Maylin, G., Duer, W., ... & Tobin, T. (2021). Dexamethasone serum concentrations after intravenous administration in horses during race training. Comparative Exercise Physiology, 17(3), 263-272.

An In-Depth Look at Stall Contamination: A Total of 28 Substances Were Identified in Charles Town Ship-In Stalls as a Mix of Human Medications and Recreational Substances with Some Actual Equine Medications

Summary: A series of low-concentration positives being 'called' in West Virginia led to an investigation into the possibility of stall contamination. Of the stalls swabbed, only four of the twenty-one tested were not considered to test 'positive' for at least a single drug – categorized as human recreational, human therapeutic, or equine therapeutic. This indicates a large chance of environmental contamination in any ship-in stall, and as testing becomes more and more sensitive it will be more and more important for thresholds to be established to avoid just such exposures resulting in a 'positive' that could otherwise end careers.

Citation: Fenger, Clara; Catignani, Maria; Machin, Jake; and Tobin, Thomas, "An In-Depth Look at Stall Contamination: A Total of 28 Substances Were Identified in Charles Town Ship-In Stalls as a Mix of Human Medications and Recreational Substances with Some Actual Equine Medications" (2017). Gluck Equine Research Center Faculty Publications. 34.
https://uknowledge.uky.edu/gerc_facpub/34

Tramadol: A Human Therapeutic Medication and an Environmental Substance Occasionally Identified in Equine Drug Testing

Abstract: Tramadol is a centrally acting synthetic opioid analgesic prescribed for acute and chronic pain in human and veterinary medicine. The human dose is 50-100 mg at 4-6 hr intervals, not to exceed 400 mg Tramadol per day. For optimal activity Tramadol must be metabolized to O-DesMethylTramadol (ODMT), which has 200-500-fold more opiate receptor activity than Tramadol. Horses rapidly metabolize ODMT to an inactive glucuronide metabolite, which renders Tramadol much less therapeutically effective in the horse. In horseracing, Tramadol is classified as an Association of Racing Commissioners International (ARCI) Class 2, Penalty Class A substance [Version 11.00 December 2015]. Tramadol is chemically stable in the environment and has been found at significant concentrations in urban wastewater.

Recently, equine drug testing laboratories have been reporting low concentration urinary ODMT 'identifications/positives', which may result in significant penalties for trainers and owners. In horses, Tramadol is metabolized to ODMT, which is then rapidly glucuronidated and excreted at high concentration in equine urine. Tramadol therefore has marginal therapeutic efficacy in horses but since Tramadol persists in the environment and its metabolite, glucuronidated ODMT, is very effectively concentrated in equine urine, Tramadol is highly likely to yield environmentally driven low concentration identifications ('positives') in the urine of horses exposed to Tramadol containing environments, as recognized in a number of jurisdictions.

Review of Tramadol/ODMT 'positives' shows a number of low concentration identifications in the urine from horses associated with individuals prescribed Tramadol. Given the medical reasons for prescribing Tramadol to humans and the marginal therapeutic efficacy of Tramadol in horses, the calling of 'positives' for pharmacologically ineffective trace concentration identifications of ODMT in post-race urine samples is difficult to justify and may selectively penalize older horsepersons who are more likely to be prescribed Tramadol for chronic pain. We believe a more equitable approach is to specify a urinary ODMT (screening or cut-off level) (we propose 50

ng/mL) below which regulatory action is not warranted, as has been done for morphine and other human associated environmental substances (Tobin et al., 2012).

Conclusion: The widespread use of Tramadol by humans, it's chemical stability in the environment, ready oral absorption by equines, marginal activity in horses, and very efficient excretion into the urine as ODMT glucuronide, it is unsurprising to find low level Tramadol 'positives' occurring sporadically. While oftentimes it can be determined to be related to a human exposure, it may also be from its persistence in the environment around a horse. As such, we recommend a 50ng/mL ODMT urinary 'cut-off' based on current reported concentration levels in 'positive' findings.

A Cluster of Trace-Concentration Methamphetamine Identifications in Racehorses Associated with a Methamphetamine-Contaminated Horse Trailer: A Report and Analysis

Abstract: Three low concentration methamphetamine "positive" tests were linked to use of a methamphetamine-contaminated trailer to transport the affected horses. This incident establishes methamphetamine as a human-use substance that can inadvertently enter the environment of racing horses, resulting in urinary methamphetamine "positives;" an interim regulatory cut-off of 15 ng/mL for methamphetamine in post-race urine is proposed.

Conclusion: Based on current regulations and the high potential for environmental contamination of methamphetamine, a 15 ng/mL threshold is proposed until further studies can determine a more appropriate cut-off. This is noticeably lower than the 500 ng/mL cut-off used by the US Department of Health and Human Services.

Citation: Brewer, K., Shults, T. F., Machin, J., Kudrimoti, S., Eisenberg, R. L., Hartman, P., ... & Tobin, T. (2016). A cluster of trace-concentration methamphetamine identifications in racehorses associated with a methamphetamine-contaminated horse trailer: a report and analysis. The Canadian Veterinary Journal, 57(8), 860.

Aminorex identified in horse urine following consumption of Barbarea vulgaris, a preliminary report

Abstract: Aminorex, (RS)-5- Phenyl-4,5-dihydro-1,3-oxazol-2-amine, is an amphetamine-like anorectic and in the United States a Drug Enforcement Administration [DEA] Schedule 1 controlled substance. Aminorex in horse urine is usually present as a metabolite of Levamisole, an equine anthelmintic and immune stimulant. Recently, Aminorex identifications have been reported in horse urine with no history or evidence of Levamisole administration. Analysis of the urine samples suggested a botanical source, directing attention to the Brassicaceae plant family, with their contained GlucoBarbarin and Barbarin as possible sources of Aminorex. Since horsepersons face up to a 1-year suspension and a $10,000.00 fine for an Aminorex identification, the existence of natural sources of Aminorex precursors in equine feedstuffs is of importance to both individual horsepersons and the industry worldwide.

Results

Testing the hypothesis that Brassicaceae plants could give rise to Aminorex identifications in equine urine we botanically identified and harvested flowering Kentucky Barbarea vulgaris, ("Yellow Rocket") in May 2018 in Kentucky and administered the plant orally to two horses. Analysis of post-administration urine samples yielded Aminorex, showing that consumption of Kentucky Barbarea vulgaris can give rise to Aminorex identifications in equine urine.

Conclusions

Aminorex has been identified in post administration urine samples from horses fed freshly harvested flowering Kentucky Barbarea vulgaris, colloquially "Yellow Rocket". These identifications are consistent with occasional low concentration identifications of Aminorex in equine samples submitted for drug testing. The source of these Aminorex identifications is believed to be the chemically related Barbarin, found as its precursor GlucoBarbarin in Kentucky Barbarea vulgaris and related Brassicaceae plants worldwide.

Experimental: Flowering Barbarea vulgaris were harvested, cleaned, and administered to horses with pre- and post-administration blood/urine test samples collected. Administration was performed by offering to horses as feed, which was initially declined.

Upon being mixed with sweet feed, however, the plant material was readily taken up by the horses. Analyses were performed via LC-MS. No aminorex was detected in the plasma samples, but urinary concentrations were estimated to be approximately 10 ng/mL.

Conclusion: "Yellow Rocket" plant (B. vulgaris) has been shown to be a causative agent in the production of detectable levels of aminorex in equine urine when consumed. Further studies are needed to elucidate the exact chemical responsible, though barbarin is a primary candidate.

Citation: Maylin, G., Fenger, C., Machin, J., Kudrimoti, S., Eisenberg, R., Green, J., & Tobin, T. (2019). Aminorex identified in horse urine following consumption of Barbarea vulgaris, a preliminary report. Irish veterinary journal, 72(1), 1-8.

BIBLIOGRAPHY

Agriculture Canada (1991) Race Track Division Schedule of Drugs. Minister of Agriculture, pp. 5 83. Agriculture Canada, Ottawa, Ontario.

Angst, F. (2018) https://www.bloodhorse.com/horse-racing/articles/227038/with-too-many-unknowns-kentucky-rescinds-drug-positive April 17, 2018. Accessed May 21, 2020

Anonymous. (2016). Canadian Pari-mutual Agency Elimination Guidelines 2016. Agriculture and Agri-Food Canada. Available at: http://www.agr.gc.ca/resources/prod/CMS/Internet/Common-Commun/1454071417865_equine_elimination_guidelines_2016-eng.pdf. Accessed August 3, 2017

Anonymous. (2017). FEI List of Detection Times. Federation Equestre Internationale. Available at: https://inside.fei.org/system/files/2017%20FEI_detection_times.pdf. Accessed August 3, 2017

Anonymous (1998) European Agency for the Evaluation of Medicinal Product. Note for Guidance for the Determination of Withdrawal Periods for Milk. European Agency for the Evaluation of Medicinal Products, Evaluation of Medicines for Veterinary Use, EMEA/CVMP/473/98 -FINAL, 11/26.

ARCI Uniform Classification Guidelines for Foreign Substances January 2018 (V.13.4).

Barker, S. A. (2009). The formation of Aminorex in racehorses following levamisole administration. A quantitative and chiral analysis following synthetic Aminorex or levamisole administration vs. Aminorex-positive samples from the field: a preliminary report. Journal of veterinary pharmacology and therapeutics, 32(2), 160-166.

Association of Racing Commissioners International. (2019). The Association of Racing Commissioners International's Model Rules of Racing, v 9.1. ARCI.com. https://www.arci.com/wp-content/uploads/2019/09/Model_Rules_MASTER_V9.1AUGUST2019.pdf.

Association of Racing Commissioners International. 2019. http://arci.com/wp-content/uploads/2019/01/2019_01_CTS_V4_1.pdf, accessed September 25, 2019.

Australian Equine Veterinary Association (1992) Detection of Substances in Racing Horses, pp. 8 49. Australian Equine Veterinary Association, Artarmon, Australia.

Baldwin, A.C., Stevenson, S.W. and Dudley, G.A., 2001. Nonsteroidal anti-inflammatory therapy after eccentric exercise in healthy older individuals. Journal of Gerontology Series A 56: M510-M513.

Barker, S.A., 2008. Drug contamination of the equine racetrack environment: a preliminary examination. Journal of Veterinary Pharmacology and Therapeutics 31: 466-471.

Barker, S. A. (2009). The formation of Aminorex in racehorses following levamisole administration. A quantitative and chiral analysis following synthetic Aminorex or levamisole administration vs. Aminorex-positive samples from the field: a preliminary report. Journal of veterinary pharmacology and therapeutics, 32(2), 160-166.

Becker, D. E., & Reed, K. L. (2006). Essentials of local anesthetic pharmacology. Anesthesia progress, 53(3), 98-109.

Bjornsd € ottir, S., Ekman, S., Eksell, P. and Lord, P. (2004) High detail radiography and histology of the centrodistal tarsal joint of Icelandic horses aged 6 months to 6 years. Equine Vet. J. 36, 5-11

Matt Bonesteel. (2021). Churchill Downs suspends Bob Baffert, the embattled trainer of Medina Spirit, for two years. June 2, 2021. *The Washington Post*

Botanical Society of Britain & Ireland. "Online Atlas of the British and Irish Flora for Barbarea vulgaris" (August 7, 2019). Online Atlas of the British and Irish Flora. Retrieved from https://www.brc.ac.uk/plantatlas/plant/barbareavulgaris on 16 May 2019. Brewer K, Dirikolu L, Hughes CG, Tobin T. (2014) Scopolamine in racing horses: trace identifications associated with dietary or environmental exposure. Vet J. 2014 Mar;199(3):324-31

Brewer, K., Shults, T. F., Machin, J., Kudrimoti, S., Eisenberg, R. L., Hartmann, P, and Tobin, T. (2016). A cluster of trace-concentration methamphetamine identifications in racehorses associated with a methamphetamine-contaminated horse trailer: A report and analysis. The Canadian Veterinary Journal, 57(8), 860.

British Horseracing Authority, 2015. Results of enquiries. (J. Doyle, Christmas Spirit, E. Williams, J. Scott, Various Trainers, A. Bailey, W. Treacy) heard by disciplinary panel on Thursday 24 September. Available at: https://tinyurl.com/yyb7zrx5

Britt B, Boyars TD. Antimicrobial drugs used by veterinarians. Proc Annu Convent AAEP. 1997; 43:170–7.

Budhraja, A., Camargo, F.C., Hughes, C., Lehner, A.F., Stirling, K., Brennan, N.,
Dowling, M., and Tobin, T., 2007. Caffeine and theobromine identifications in post-race urines: threshold levels and regulatory significance of such identifications. AAEP Proceedings 53: 87-92.

Cady, Steve (1970). Kentucky Court Awards First-Place Money in 68 Derby to Dancer's Image. New York Times, December 12, 1970

Cagnardi, P., Gallo, M., Zonca, A., Carli, S. and Villa, R., 2011.Pharmacokinetics and
effects of alkalization during oral and intravenous administration of naproxen in horses. Journal of Equine Veterinary Science 31: 456-462.

California, S. O. (n.d.). Query Rules & Regulations Results. Retrieved March 01, 2018, from http://www.chrb.ca.gov/query_rules_and_regulations_database.asp?form_query_action=display_rule&form_query_rule_number=1844&form_query_rule_title=Authorized%2BMedication.&form_query_article=Authorized%2BMedication.&form_query_article_index=17&form_query_argument=1844Camargo, F.C., Hughes, C., Lehner, A.F. and Tobin, T., 2006. Trace level benzoylecgonine identifications in post-race urines: probable sources and the regulatory significance of such identifications. AAEP Proceedings 52: 331-336.

Camargo, F.C., Lehner, A.F., Karpiesiuk, W., Stirling, K., Kavanagh, P.V., Brennan, N., Dowling, M. and Tobin, T., 2005. Review of environmental morphine identifications: worldwide occurrences and responses of authorities. AAEP Proceedings 51: 58-64.

Canadian pari-mutuel Agency. 2016. http://www.agr.gc.ca/resources/prod/CMS/Internet/Common-Commun/1454071417865_equine_elimination_guidelines_2016-eng.pdf, accessed September 25, 2019.

Choo H.Y. P. & Choi S. O. (1991) The metabolism of xylazine in rats Anal Pharm Res 14(4), 346-251

Christine, Bill. (1988). The 1968 Kentucky Derby Victory That Wasn't: Dancer's Image, who Finished First, was Disqualified After Positive Test for Illegal Medication. *Los Angeles Times*

Clayton, H.M., Sha, H.M., Stick, J.A. and Robinson, P. (2007) 3D kinematics of the interphalangeal joints of walking and trotting horses. Vet. Comp. Orthop. Traumatol. 20, 1-7.

Crawley, M.J. (2015) Statistics: An introduction using R, 2nd edn., John Wiley & Sons Ltd, Chichester, West Sussex, UK. pp 234-237.

Davies, N.M. and Anderson, K.E., 1997. Clinical pharmacokinetics of naproxen. Clinical Pharmacokinetics 32: 268-293.

Dorsey, N. E. (1944). The velocity of light. Transactions of the American Philosophical Society, 34(1), 1-110.

Dudley, G.A., Czerkawski, J., Meinrod, A., Gillis, G., Baldwin, A. and Scarpone, M., 1997. Efficacy of naproxen sodium for exercise-induced dysfunction muscle injury and soreness. Clinical Journal of Sport Medicine 7: 3-10.

Eiden, C., Peyrière, H., Diot, C., & Mathieu, O. (2015). Prevalence of levamisole and Aminorex in patients tested positive for cocaine in a French University Hospital. Clinical toxicology, 53(7),604-608

Eisenberg, R., Kudrimoti, S., Hughes, C.G., Maylin, G.A. and Tobin, T. (2014) Synthesis, purification, and chemical characterization of 20- dihydro-6-methylprednisone, an isomeric metabolite of methylprednisolone in the horse, for use as an analytical standard. Drug Test. Anal. 6, 303-307

European Horserace Scientific Liaison Committee (1997) Information for Veterinary Surgeons on Detection Periods of Named Drugs, R & W Publications Limited, Newmarket, UK.

Federation Equestrian Internationale. 2018. https://inside.fei.org/system/files/FEI%20Detection%20Times%202018_0.pdf, accessed September 25, 2019.\

Frakes, Jason. (2021). Bob Baffert acknowledges using ointment on Medina Spirit that includes betamethasone. May 11, 2021. *Louisville Courier Journal*

Furlan L, et al. The efficacy of biofumigant meals and plants to control wireworm populations. Ind Crop Prod. 2010;31(2):245–54.

Gabrielsson JL, & Weiner DL. 1999. Methodology for pharmacokinetic/ pharmacodynamic data analysis. Pharmaceutical science & technology today, 2(6), 244-252.

Garcia V. R., Toutain P. L., Alveinerie M. & Ruckebusch Y. (1981) The pharmacokinetics of xylazine hydrochloride: an interspecific study Journal of Veterinary Pharmacology and Therapeutics 4, 87–92

Greene S. A. & Thurmon J. C. (1988) Xylazine – a review of its pharmacology and use in veterinary medicine Journal of Veterinary Pharmacology and Therapeutics 11, 295–313

Gutierrez, J., Eisenberg, R. L., Koval, N. J., Armstrong, E. R., Tharappel, J., Hughes, C. G., &Tobin, T. (2010). Pemoline and Tetramisole 'positives' in English racehorses following levamisole administration. Irish veterinary journal, 63(8), 498.

Hackett, George W. (May 8, 1968). "K-Derby's Dancer Disqualified". The Tampa Tribune. AP. p. 19. Retrieved August 2021 – via newspapers.com.

Harkins, J. D., Karpiesiuk, W., Woods, W. E., Lehner, A., Mundy, G. D., Rees, W. A., ... & Tobin, T. (1999). Mepivacaine: its pharmacological effects and their relationship to analytical findings in the horse. Journal of veterinary pharmacology and therapeutics, 22, 107-121

Helsel, D. R. (2005). Nondetects and data analysis. Statistics for censored environmental data. Wiley-Interscience.

Helsel, D.R. (2012) Robust imputation and NADA (Nondetects and Data Analysis for R Software). In: Statistics for Censored Environmental Data Using Minitab_ and R, 2nd edn., Wiley and Sons, Hoboken, NJ. pp 79-98 and 297–302

Ho, E. N. M., Leung, D. K. K., Leung, G. N. W., Wan, T. S. M., Wong, A. S. Y., Wong, C.H.F., ... & Sams, R. (2009). Aminorex and rexamino as metabolites of levamisole in the horse. Analytica chimica acta, 638(1), 58-68.

Hofmaier, T., Luf, A., Seddik, A., Stockner, T., Holy, M., Freissmuth, M., ... & Kudlacek, O. (2014). Aminorex, a metabolite of the cocaine adulterant levamisole, exerts amphetamine like actions at monoamine transporters. Neurochemistry international, 73, 32-41.

Holloway, K, (2019) Aminorex and Pemoline Identifications in US Racing: Personal communication from the Association of Racing Commissioners International, 2/20/2019.

Jeffcott, L.B., Rossdale, P.D., Freestone, J., Frank, C.J. and Towers-Clark, P.F. (1982) An assessment of wastage in Thoroughbred racing from conception to 4 years of age. Equine Vet. J. 14, 185-198.

Johnstone, I.B., 1983. Comparative effects of phenylbutazone, naproxen and flunixin meglumine on equine platelet aggregation and platelet factor 3 availability in vitro. Canadian Journal of Comparative Medicine 47: 172-179.

Kabacoff, R.I. (2015) R in Action, 2nd edn., Manning Publications Co., Shelter, NY. pp 222-223.

KHRC. (2015). Kentucky Horse Racing Commission Withdrawal Guidelines Thoroughbred; Standardbred; Quarter Horse, Appaloosa, and Arabian KHRC 40-02. http://khrc.ky.gov/Documents/KHRC%2040-02-Withdrawal%20Guidelines.pdf (accessed Nov 27, 2016).

Kjaer, A., & Gmelin, R. (1957). IsoThiocyanates. XXVII. A New isoThyiocyanate Glucoside (GlucoBarbarin) Furnishing (-)-5-Phenyl-2-oxazolidinethione upon Enzymic Hydrolysis. L. Acta Chemica Scandinavica, 11, 906-907. doi: 10.3891/acta.chem.scand.11- 0906

Kjaer, A., & Gmelin, R. (1958). IsoThiocyanates. XXXIII. An isoThyiocyanate Glucoside (GlucoBarbarin) of Reseda luteola L. Acta Chemica Scandinavica, 12, 1693-1694. Doi: 10.3891/acta.chem.scand.12-1693

Knych, H.K., Blea, J.A., Arthur, R.M., Overly, L.R. and McIlwraith, C.W. (2016) Clearance of corticosteroids following intra-articular administration of clinical doses to racehorses. Equine Vet. Educ. 28, 140-144.

Knych, H.K., Harrison, L.M., Casbeer, H.C. and McKemie, D.S. (2014) Disposition of methylprednisolone acetate in plasma, urine, and synovial fluid following intra-articular administration to exercised Thoroughbred horses. J. Vet. Pharmacol. Ther. 37, 125-132

Knych HK, Stanley SD, Harrison LM, Mckemie DS. 2017. Pharmacokinetics of betamethasone in plasma, urine, and synovial fluid following intra-articular administration to exercised thoroughbred horses. Drug Test Anal. Sep;9(9):1385-1391. doi: 10.1002/dta.2170. Epub 2017 Mar 7.

Kollias-Baker C, Cox K, Jones J. Evaluation of the effects of omeprazole on physiological indices of performance of horses during incremental treadmill exercise. Vet Ther. 2001 Fall;2(4):361-9. PMID: 19746659.

Kudrimoti S., Eisenberg R., Maylin G. A., Hughes C. & Tobin.T. (2014) Synthesis and characterization of 4-hydroxyxylazine and 4-hydroxyxylazine-d6 standards for use in xylazine regulation in competition horses Proc 20th Int Conf Racing Anal Vet, 332-335

Lanier, A.B., 2003. Use of nonsteroidal anti-inflammatory drugs following exercise induced muscle injury. Sports Medicine 33:177-186.

Lees, P. and Higgins, A.J., 1985. Clinical pharmacology and therapeutic uses of non-steroidal anti-inflammatory drugs in the horse. Equine Veterinary Journal 17: 83-96.

Loganathan, D., Fu, D., Zhao, S., Kwan, R., Chen, E., & Morrissey, B. (2009). Occurrence of Aminorex and Levamisole in Canadian Racing Part 2: Comparison of Levamisole and Aminorex Metabolism and Elimination Profiles in the Horse. In Proceedings of the 17th International Conference of Racing Analysis and Veterinarians. Antalya, Turkey.

Machin, J. K. Brewer, M. Catignani T.F. Shults C. Fenger G.A. Maylin and T. Tobin (2019) An interim screening limit of detection for naproxen in equine plasma: a review and analysis; Comparative Exercise Physiology,2019,16,153-160.

Machin, J., Duer, W., Maylin, G., Fenger, C., Wilson, D., Ivey, M., ... & Tobin, T. (2019). Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: A field study. Equine veterinary journal, 51(3), 343-348.

Machin, J. Kudrimoti, S. Eisenberg, R., Fenger, C., Hartmann, P., Maylin, G., Tobin, T., and Shults, T. (2018). "Synthesis, Characterization and Certification of Barbarin, a Possible Botanical Source of Aminorex Identifications". Submitted for presentation and publication, the Society of Forensic Toxicology (SOFT) Annual Meeting, Oct 07-12, 2018. Minneapolis, Minnesota. p 92

Machin, J., Shults, T., Fenger, C. and Tobin, T., 2017. Inadvertent environmental transfer of dextromethorphan from groom to racehorse results in establishment of a pharmacologically relevant cutoff. Toxtalk 41: 20-23.

Maindonald, J., & Braun, J. (2006). Data analysis and graphics using R: an example-based approach (Vol. 10). Cambridge University Press.

Managan, Dan. "Kentucky Derby Winner Medina Spirit's Failed Drug Test Is Confirmed." Cnbc.com, CNBC, 2 June 2021, 1044, www.cnbc.com/2021/06/02/kentucky-derby-winner-medina-spirits-failed-drug-test-is-confirmed.html.

Maylin, G., Fenger, C., Machin, J., Kudrimoti, S., Eisenberg, R., Green, J., & Tobin, T. (2019). Aminorex identified in horse urine following consumption of Barbarea vulgaris, a preliminary report. Irish Veterinary Journal, 72(1), 15.p2-8

Menendez MI, Phelps MA, Bertone AL. 2016. Pharmacokinetics of intra-articular betamethasone sodium phosphate and betamethasone acetate and endogenous hydrocortisone suppression in exercising horses. J Vet Pharmacol Ther. Feb;39(1):22-6. doi: 10.1111/jvp.12229. Epub 2015 Apr 3.

Norgren, A., Ingvast-Larsson, C., Kallings, P., Fredriksson, F. and Bondesson, U., 2000.

Contamination and urinary excretion of flunixin after repeated administration in the horse. In: Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R&W, Cambridge, UK, pp. 377-380.

Norman, JJ, Heggie, RM, and Larose JB, 1962 Oximes: 1. The synthesis of some substituted 2-oximinoacetophenones Canadian Journal of Chemistry, 40(8): 1547-1553, https://doi.org/10.1139/v62-233

Owen, D. B. (1962). Handbook of statistical tables (No. R HA48 O9).

Paulick Report Staff. (2021). "Report: Why Regulators Test for Picograms of Betamethasone". https://www.paulickreport.com/horse-care-category/report-why-regulators-test-for-picograms-of-betamethasone/. Accessed June 5, 2021.

Preston, S.A., Zimmel, D.N., Chmielewski, T.L., Trumble, T.N., Brown, M.P., Boneau, J.C. and Hernandez, J.A. (2010) Prevalence of various presale radiographic findings and association of findings with sales price in Thoroughbred yearlings sold in Kentucky. J. Am. Vet. Med. Ass. 236, 440-445.

Poos, G. I., Carson, J. R., Rosenau, J. D., Roszkowski, A. P., Kelley, N. M., & McGowin, J. (1963). 2-Amino-5-aryl-2-oxazolines. Potent new anorectic agents. Journal of medicinal chemistry, 6(3), 266-272.

Popot, M.A., Garcia, P. and Bonnaire, Y., 2011. Doping control in horses: housing conditions and oral recycling of flunixin by ingestion of contaminated straw. Journal of Veterinary Pharmacology and Therapeutics 34: 612-614.

RMTC. 2016. http://rmtcnet.com/wp-content/uploads/2016/02/Explaining-the-95-95-Tolerance-Interval.pdf, accessed September 3, 2021.

RMTC (Racing Medication and Testing Consortium). 2019. https://rmtcnet.com/wp-content/uploads/RMTC-Betamethasone-Monograph-Jan-2019.pdf, accessed September 25, 2019.

Rumpler, M.J., Colahan, P. and Sams, R.A. (2014) The pharmacokinetics of glycopyrrolate in Standardbred horses. J. Vet. Pharmacol. Ther. 37, 260 -268.

Russell, C.S. and Maynard, S., 2000. Environmental contamination with isoxsuprine. In: Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R&W, Cambridge, UK, pp. 381-383.

Sams, R.A. (2006) Detection and Identification of Aminorex and Rexamino from Blood and Urine Samples from Racehorses. In Proceedings of the 16th International Conference of Racing Analysts and Veterinarians. Tokyo, Japan.

Santoro, R., Warren, R., & Roberts, G. (1976). Spontaneous formation of 4-methyl-5-phenyloxazolidine-2-thione from phenylpropanolamine. Journal of Chromatography A, 117(2),375-382.

Savage, I. R. (1961). Probability inequalities of the Tchebycheff type. Journal of Research of the National Bureau of Standards-B. Mathematics and Mathematical Physics B, 65(3), 211-222.

Savage, R. (2016) Probability inequalities of the Tchebycheff (Chebyshev) type. J. Res. Natl. Bur. Stand. 65B, 211-222.

Snyder, S.A., 2008. Occurrence, treatment, and toxicological relevance of EDCs and pharmaceuticals in water. Ozone: Science and Engineering 30: 65-69.

Soma, L. R., Rudy, J. A., Uboh, C. E., Xu, F., & Snapp, H. M. (2008). Pharmacokinetics and effects of Aminorex in horses. American Journal of Veterinary Research, 69(5), 675-681.

Soma, L.R., Uboh, C.E., Rudy, J.A. and Perkowski, S.Z., 1995. Plasma and synovial

fluid kinetics, disposition and urinary excretion of naproxen in horses. American Journal of Veterinary Research 56: 1075-1080.

Soma LR, Rudy JA, Udoh CE, Xu F, Snapp HM. Pharmacokinetics and effects of Aminorex in horses. Am J Vet Res. 2008;69(5):675–81.

Spyridakia M. H., Lyrisa E., Georgoulakisb I., Kouretasb D., Konstantinidouc M. & Georgakopoulosa C.G. (2004) Determination of xylazine and its metabolites by GC–MS in equine urine for doping analysis J Pharm Biomed Analysis 35(1), 107-116.

Stevenson, A.J., Weber, M.P., Hopkins, D. and Kacew, S., 1997. Anti- inflammatory drugs. Analytical methodology for detection and confirmation of drugs in equine body fluids. Vol. III. Canadian Equine Drug Evaluation Program – Canadian Pari-Mutuel Agency, Agriculture and Agri-Food Canada, Ottawa, ON, Canada, pp. 194-207.

Teale, P. and Biddle, S. (2018). Possible Plant Sources of Aminorex and Potential markers of Their Ingestion in the Horse. Proceeds of the 22nd International Conference of Racing Analysists and Veterinarians. Dubai, UAE in press.

Tobin, T. (1998). Detection and Quantitation of 3-Hydroxymepivacaine in Horse Urine: A Preliminary Report. Proceedings of the 12th International Conference of Racing Analysts and Veterinarians, 324-328.

Tobin T., Brewer K. & Stirling K. H. (2012) World Rules for Equine Drug Testing and Therapeutic Medication Regulation. Nicholasville, Kentucky. Wind Publications.

Tobin T. & Charles C. T. (1981) Drugs and the Performance Horse, Charles C Thomas Pub Ltd ISBN-10: 0398044465

Tobin, T., Dirikolu, L., Brewer, K., & Hughes, C. G. (2013). A clinician's guide to factors affecting withdrawal times for equine therapeutic medications. *The Veterinary Journal*, 198(2), 313-321.

Tobin, T., Harkins, J. D., & Sams, R. A. (1999). Testing for therapeutic medications: analytical/pharmacological relationships and 'limitations' on the sensitivity of testing for certain agents. Journal of veterinary pharmacology and therapeutics, 22(3), 220-233.

Toutain P. L. (2010). How to extrapolate a withdrawal time from an EHSLC published detection time: a Monte Carlo simulation appraisal. Equine veterinary journal, 42(3), 248-254.

Toutain, P.L. and Lassourd, V., 2002a. Pharmacokinetic/pharmacodynamic approach to assess irrelevant plasma or urine drug concentrations in postcompetition samples for drug control in the horse. Equine Veterinary Journal 34: 242-249.

Toutain, P.L. and Lassourd, V., 2002b. Pharmacokinetic/pharmacodynamics assessment of irrelevant drug concentrations in horse plasma or urine for a selection of drugs. In: Proceedings of the 14th International Conference of Racing Analysts and Veterinarians.Orlando, FL, USA.

United States Equestrian Federation. 2019. https://www.usef.org/forms-pubs/2Zp2C_YKs4s/2019-equine-drugs-medications, accessed September 25, 2019.

USDA. "Plants Profile for Barbarea vulgaris." Plants Profile for Barbarea vulgaris (Yellow Rocket), United States Department of Agriculture, plants. usda.gov/core/profile? Symbol. Accessed May 24, 2018.

Voss N, (2018) https://www.paulickreport.com/news/the-biz/the-trouble- with-aminorex/ April 17, 2018. Accessed March 21, 2020

Wagner A. E., Muir W. W. & Hinchcliff K. W. (1991) Cardiovascular effects of xylazine and detomidine in horses. Am J Vet Res 52(5), 651-657

Walter, Jr, C R 1952 Preparation of Primary Amines by Reduction of Oximes with Lithium Aluminum Hydride and by the Leuckart Reaction, Journal of American Chemical Society,74(20),5185-5187.

Wennerlund, I., Ingvast-Larsson, C., Kallings, P., Fredriksson, E. and Bondesson, U., 2000. Pharmacokinetics and urinary excretion of naproxen after repeated oral administration. In: Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R&W, Cambridge, UK, pp. 195-200.

Whitmore, Dennis, 2017 Horseplop.com, Harness Racing, Aminorex, November 28th, 2017.

Williams, R.B., Woodward, K., Hines, S. and Teale, P., 2000. Urinary detection of ibuprofen: feed contamination, topical application and oral administration. In: Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R&W, Cambridge, UK, pp. 372-376.

World Anti-Doping Agency (WADA), 2017. Decision limits for the confirmatory quantification of threshold substances. WADA Technical Document – TD2017DL. Available at: https://tinyurl.com/y6y5yna7.

World Anti-Doping Agency (WADA), 2019. What is prohibited. Available at: https://tinyurl.com/y7fyyqkr.

Zuccato, E., Calamari, D., Natangelo, M. and Fanelli, R., 2000. Presence of therapeutic drugs in the environment. The Lancet 355: 1789-1790.

VITA OF JACOB JOSEPH MACHIN

Educational Institutions attended and degrees awarded:

B.S. (Chemistry), College of William and Mary, Williamsburg, VA, USA, 2011

UK Doctoral Degree (Toxicology and Cancer Biology), Univ. of KY, Lexington, KY, USA, 2015-Present, Advisor: Thomas Tobin

Professional positions held:

USEF Laboratory Assistant (July-September 2018) under Dr. Cornelius Uboh

Invited Seminars, Presentations, and Related Events

1) National HBPA Summer Meeting, Denver, Colorado, Saturday, August 8th, 2015: Glenys K. Noble, Kimberly Brewer, MaryAnn O'Connell, K. H. Stirling, A. Morales Briceno, J. Machin, and C. Hughes. T. Tobin: "Revisiting the Racing Medication and Testing Consortium [RMTC] Xylazine Guidelines, with Australian Assistance; Presentation to the Summer 2015 Medication Forum of the National Horsemen's Benevolent and Protective Association Meeting, Denver, Colorado, Saturday, August

8th 2015.

2) K. Brewer, J. Machin, K. Stirling, Abelardo Morales Briceno, C. Hughes and T. Tobin. Canadian Environmental Methamphetamines Identifications Linked to a Horse Trailer: Presentation to the Summer 2015 Medication Forum of the National Horsemen's Benevolent and Protective Association Meeting, Denver, Colorado, Saturday, August 8th, 2015.

3) Tobin T, Invited presentation to the National Horsemen's Benevolent and Protective Association, Winter Convention, Clearwater, Florida, "Inconsequential Trace Level

Identifications of Environmental Substances" K. H. Stirling, K. Brewer, MaryAnn O'Connell, A. Morales Briceno, J. Machin and T. Tobin. February 3-6-7th, 2016.

4) CATHINONE: A Human Recreational Substance Identified in Post-Race Urines Presentation to Azam Syed, Pharmacy in Charge, Zabeel Palace, Dubai and The A Team, Jake Machin, Kimberly Brewer, Sucheta Kudrimoti, Kent H. Stirling, Rodney Eisenberg and Thomas Tobin Marmoom Equine Research Center, Marmoom,

Dubai UAE, Wednesday, April 6th, 2016.

5) "EQUINE MEDICATION TESTING: 100 YEAR AND STILL LEARNING" Thomas Tobin, ShivaKumar Gudlawar, Jake Machin, Kimberly Brewer, Sucheta Kudrimoti, Rodney Eisenberg and Kent H. Stirling the Maxwell H. Gluck Equine Research Center, University of Kentucky, The Marmoom Equine Research Center, Marmoom, Dubai, UAE, The Florida Horsemen's Benevolent and Protective Association; Presented to The Anti-Doping Laboratory Qatar [ADLQ] Annual Meeting entitled "ANTI-DOPING IN

HUMANS AND ANIMALS: PARALLELS AND DIVERGENCES" in conjunction with the World Anti-Doping Association [WADA] at the Doha Marriott Hotel, Qatar, Wednesday, June 1st, 2016.

6) Jake Machin, Kimberly Brewer, Thomas Tobin, "MATTER OF DURING OR POST COLLECTION CONTAMINATION: AN OVERVIEW OF SOME CASES" National Horsemen's Benevolent and Protective Association, Summer Convention, Vancouver,

Canada, July 16th, 2016.

7) Kevin Kersh, DVM, DACVS; Scott R. McClure, DVM, PhD, DACVS, DACVSMR; Jake Machin, MS; Levent Dirikilou, PhD; Brad Brown, DVM; Clara Fenger, DVM, PhD, DACVIM; George Maylin, DVM, PhD; Edward Roualdes, PhD; Thomas Tobin, MRCVS, PhD, DABT Determining viable withdrawal times for dexamethasone in horses in a racing environment, where self-contamination is likely: Clinical Guidelines for application in a regulatory environment. Accepted for presentation and publication at the 21st International Conference of Racing Analysts and Veterinarians, Montevideo, Uruguay, October 15-22nd, 2016.

8) Thomas Tobin, Jake Machin, Sucheta Kudrimoti, and Rodney Eisenberg Synthesis and Certification of a Deuterated Internal Standard for Xylazine: Accepted for presentation and publication at the 21st International Conference of Racing Analysts and

Veterinarians, Montevideo, Uruguay, October 15-22nd, 2016.

9) Jacob Joseph Machin, Sucheta Kudrimoti, Rod Eisenberg, Clara Fenger, Petra Hartmann, George Maylin, Theodore Shults, and Thomas Tobin Synthesis and Characterization of Barbarin, A Potential Plant Source of Aminorex Identifications in Racing Horses (poster) at the Society of Forensic Toxicology

10) George Maylin, Clara Fenger, Jacob Joseph Machin, Sucheta Kudrimoti, Rod Eisenberg, JD Green, Thomas Tobin Aminorex Identified in Horse Urine Following Consumption of Barbarea Vulgaris (poster) at the University of Kentucky Depart of Toxicology and Cancer Biology New Student Orientation

PUBLICATIONS:

1) Kimberly Brewer, Theodore F. Shults, Thomas Tobin, Sucheta Kudrimoti, Rodney L. Eisenberg, Petra Hartman, Caroline Wang, Charlie Hughes, Jacob Joseph Machin, and Pierre Beaumier A Cluster of Trace-Concentration Methamphetamine Identifications in Racehorses Associated with a Methamphetamine-contaminated Horse Trailer: A Report and Analysis: Accepted for publication, Canadian Veterinary Journal, October 2015. Kentucky Agricultural Experiment Station (KAES Publication # 15-14-057). Equine publication # 424

2) Everett Macomber, Glenys Noble, Kimberly Brewer, Jake Machin, Abelardo Morales Briceño, Mary Ann O'Connell, Kent Stirling, Rodney Eisenberg, S Kudrimoti, Clara Fenger, and Thomas Tobin Regulatory Thresholds for Xylazine; A Case Report and Review Based on Recent Regulatory Events: Submitted for publication, Canadian Veterinary Journal.

3) Kimberly Brewer, Jake Machin, Theodore F. Shults, Gregory A. Hood, Charlie G. Hughes, Clara Fenger and Thomas Tobin. Tramadol: A Human Therapeutic Medication

and an Environmental Substance Occasionally Identified in Equine Drug Testing Final draft publication, British Veterinary Journal

4) Kevin Kersh, DVM, DACVS; Scott R. McClure, DVM, PhD, DACVS, DACVSMR; Jake Machin, MS; Levent Dirikilou, PhD; Brad Brown, DVM; Clara Fenger, DVM, PhD, DACVIM; George Maylin, DVM, PhD; Edward Roualdes, PhD; Thomas Tobin, MRCVS, PhD, DABT Determining viable withdrawal times for dexamethasone in horses in a racing environment, where self-contamination is likely: Clinical Guidelines for application in a regulatory environment. Presented and accepted for publication at the 21st International Conference of Racing Analysts and Veterinarians, Montevideo, Uruguay, October 15-22nd, 2016.

5) Thomas Tobin, Jake Machin, Sucheta Kudrimoti and Rodney Eisenberg Synthesis and Certification of a Deuterated Internal Standard for Xylazine: Presented and accepted for publication at the 21st International Conference of Racing Analysts and Veterinarians, Montevideo, Uruguay, October 15-22nd, 2016.

6) Machin J, Shults, T Fenger C and Tobin T, 2017: Inadvertent Environmental Transfer of Dextromethorphan from Groom to Racehorse results in Establishment of a Pharmacologically relevant Cutoff; ToxTalk, Vol 41 Issue#2 p 20 -23.

7) W.C. Duer, J. Machin, G. Maylin, C. Fenger, T. Tobin "APPLICATION OF ROBUST REGRESSION ON ORDER STATISTICS AND DISTRIBUTION FREE STATISTICS IN THRESHOLD DETERMINATIONS FOR THERAPEUTIC MEDICATIONS, USING METHYLPREDNISOLONE AS AN EXAMPLE" Accepted for presentation and publication, 22nd International Conference of Racing Analysts and Veterinarians, Dubai, United Arab Emirates March 3-10th 2018. KAES Manuscript Number 18-14-19. Submitted for publication

8) J Machin, W Duer, G Maylin, C Fenger, D Wilson, M Ivey, B Berthold, S Allison, T Tobin Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: a field study. Equine Veterinary Journal ISSN 0425-1644. DOI: 10.1111/evj.13003 (accepted for publication)

9) George Maylin, Clara Fenger, Jacob Joseph Machin, Sucheta Kudrimoti, Rod Eisenberg, JD Green, Thomas Tobin Aminorex Identified in Horse Urine Following Consumption of Barbarea Vulgaris Submitted to the Irish Veterinary Journal update for print information

10) Jacob Joseph Machin, Kimberly Brewer, Maria Catignani, Theodore F. Shults, Clara Fenger, G.A. Maylin, Thomas Tobin. An interim Screening Limit of Detection for Naproxen in Equine Plasma; A Review and Analysis. Submitted to the Equine and Comparative Exercise Physiology Journal. Update for print information

11) Jacob Joseph Machin, G.A. Maylin, C.K. Fenger, R. Hunt, K.E. Bladon, O. Sacopulos, Thomas Tobin. Pharmacokinetic Studies using a Highest No Effect Dose (HNED) and a Full Clinical dose of Mepivacaine in Thoroughbred Horses. Presented at the 21st International Conference of Racing Analysts and Veterinarians and under review for publication.

12) Jacob Joseph Machin, George Maylin, Wayne Duer, Clara Fenger, Thomas Tobin. Pharmacokinetics of betamethasone following intra-articular injection of 15mg of BTM sodium phosphate/acetate prodrug combination into each medial femorotibial joint of Standardbred horses. Submitted to Veterinary Pharmacology and Therapeutics

13) Jacob Joseph Machin, Sucheta Kudrimoti, Rod Eisenberg, Clara Fenger, Petra Hartmann, George Maylin, Theodore Shults, Thomas Tobin Synthesis and Characterization of Barbarin, A possible Plant Source of Aminorex Identifications in Racing Horses. Accepted, Drug Testing and Analysis

14) Jacob Joseph Machin, Taylor G. Childers, Sucheta Kudrimoti, Rodney Eisenberg, Clara Fenger, George Maylin, and Thomas Tobin, Synthesis and Characterization of d5-Barbarin for Use in Barbarin Related Research. Submitted for publication to Synthetic Communications. May 19, 2020

Jacob Joseph Machin