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MINNESOTA RACING COMMISSION

March 15, 2012

Legislative Reference Library
645 State Office Building
100 Constitution Avenue
St Paul, MN 55155

Re: In The Matter of the Proposed Rules of Racing Commission Relating to M.R.
7876 Stabling, 7890 Horse Medication, and 7897 Prohibited Acts, Governor's Tracking
#AR 1049

Dear Librarian:

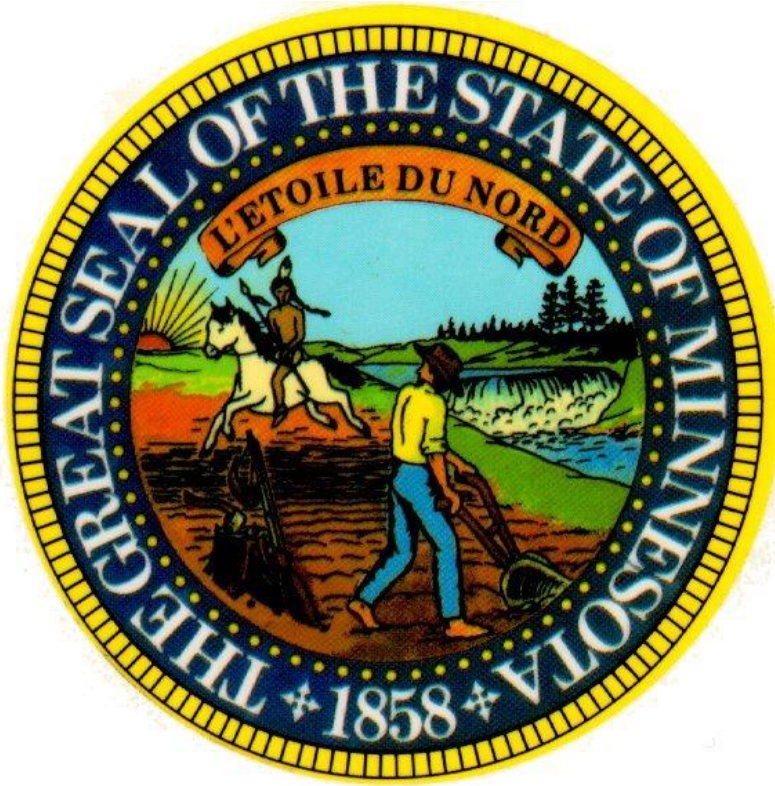
The Minnesota Racing Commission intends to adopt rules relating to Horse Medication. We plan to publish a Notice of Intent to Adopt in the March 19, 2012 State Register.

The Commission has prepared a Statement of Need and Reasonableness. As required by Minnesota Statutes, sections 14.131 and 14.23, the Commission is sending the Library a copy of the Statement of Need and Reasonableness at the same time we are mailing our Notice of Intent to Adopt Rules. I am unable to send the SONAR as an attachment because it is so large. I am sending a link to our docket that is posted on our web site. [Docket](#)

If you have questions, please contact me at 952-496-7950.

Sincerely,

Marlene Swanson
Rules Coordinator
Minnesota Racing Commission



RULEMAKING DOCKET

MINNESOTA RACING COMMISSION

February 9, 2012

If you would like to be put on a rulemaking mailing or e-mailing list, please contact Marlene Swanson at the Minnesota Racing Commission, PO Box 630, Shakopee, MN 55379 or marlene.swanson@state.mn.us.

POSSIBLE AMENDMENT TO RULES GOVERNING:

Minnesota Rule Chapter 7876 – Stabling

Minnesota Rule Chapter 7890 – Horse Medication

Minnesota Rule Chapter 7897 – Prohibited Acts

Agency Contact Person:

Richard G. Krueger, Executive Director

Minnesota Racing Commission

P.O. Box 630

Shakopee MN 55379

612-496-7950

richard.krueger@state.mn.us

or

Marlene Swanson, Rules Coordinator

Minnesota Racing Commission

P. O. Box 630

Shakopee, MN 55379

952-496-7950

marlene.swanson@state.mn.us

Subject Matter of the Proposed Rules:

The Racing Commission at its October 20, 2011 meeting, signed an Authorizing Resolution authorizing Richard Krueger, Executive Director, to request comments to rule amendments governing **Minnesota Rules, 7876 Stabling, 7890 Horse Medication, and 7897 Prohibited Acts**. The rule amendments process on these rules, started last year, but was withdrawn due to a technical problem within the Statement of Need and Reasonableness. The technical problem was pointed out by the Administrative Law Judge. The Commission is resubmitting these same rule amendments with the addition of a change to length of time a Certificate of Veterinary Inspection is valid for shipping in and out of a Minnesota racetrack.

The Racing Commission is considering rule amendments that require a record of a negative test for Equine Piroplasmosis, adds or revises definitions to Horse Medication definitions, sets regulatory limits on medications, and prohibits acts with regard to feed and medication. The requirement for a negative test for Equine Piroplasmosis is in response to an outbreak of piroplasmosis at a track in Texas and the positive test of one horse for piroplasmosis at Canterbury Park in 2010. The one positive test caused the quarantine of an entire barn. The other part of this rule amendment has been a collaborative effort on the part of the Commission and staff, the horsemen and their representative groups to provide better guidance in the administration of medications to racing horses.

Citation to all published notices relating to the proceeding:

The [Request for Comments](#) published in the November 21, 2011 issue of the State Register.

[SONAR](#)

Where written comments on the proposed rule may be inspected:

Minnesota Racing Commission

1100 Canterbury Road

Shakopee MN 55379

Minnesota Racing Commission

REQUEST FOR COMMENTS

Possible Amendment to Rules Governing Horse Racing, *Minnesota Rules*, 7876 Stabling, 7890 Horse Medication, and 7897 Prohibited Acts

Subject of Rules. The Minnesota Racing Commission requests comments on its possible amendment to rules governing Horse Racing. The Commission is considering rule amendments that require a record of a negative test for Equine Piroplasmiasis, extend the time a Certificate of Veterinary Inspection can be used after the initial entry to a racetrack, add or revise definitions to Horse Medication definitions, set regulatory limits on medications, and prohibit acts with regard to feed and medication.

Persons Affected. The amendment to the rules would likely affect Class A & B Licensees, Class C licensees, and the betting public, and horsemen.

Statutory Authority. *Minnesota Statutes*, sections 240.03, 240.24, and 240.25, authorize the Racing Commission to amend rules which affect the integrity of racing or the public health, welfare, or safety.

Public Comment. Interested persons or groups may submit comments or information on these possible rules in writing or orally until further notice is published in the *State Register* that the Commission intends to adopt or to withdraw the rules. The Commission does not contemplate appointing an advisory committee to comment on the possible rules.

Rules Drafts. The Commission has prepared a draft of the possible rules amendments.

Agency Contact Person. Written or oral comments, questions, requests to receive a draft of the rules, and requests for more information on these possible rules should be directed to:

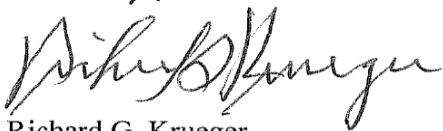
Richard G. Krueger, Executive Director
Minnesota Racing Commission
P. O. Box 630
Shakopee, MN 55379
952-496-7950
Fax 952-496-7954
Email richard.krueger@state.mn.us.

TTY users may call the Commission at 800-627-3529.

Alternative Format. Upon request, this Request for Comments can be made available in an alternative format, such as large print, Braille, or cassette tape. To make such a request, please contact the agency contact person at the address or telephone number listed above.

NOTE: Comments received in response to this notice will not necessarily be included in the formal rulemaking record submitted to the administrative law judge if and when a proceeding to adopt rules is started. The agency is required to submit to the judge only those written comments received in response to the rules after they are proposed. If you submitted comments during the development of the rules and you want to ensure that the Administrative Law Judge reviews the comments, you should resubmit the comments after the rules are formally proposed.

Dated: 11/1/11

A handwritten signature in black ink, appearing to read "Richard G. Krueger". The signature is written in a cursive style with some loops and flourishes.

Richard G. Krueger

Minnesota Racing Commission

MINNESOTA RACING COMMISSION

STATEMENT OF NEED AND REASONABLENESS

12/20/2011

Upon request, this Statement of Need and Reasonableness can be made available in an alternative format, such as large print, Braille, or cassette tape, or digital disc. To make a request, contact Ms. Marlene Swanson at the Minnesota Racing Commission, P. O. Box 630, Shakopee, MN 55379; phone 952-496-7950, fax 952-496-7954; or email at marlene.swanson@state.mn.us. TTY users may call the Racing Commission at 800-627-3529.

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INTRODUCTION

The Racing Commission is considering rule amendments that require a record of a negative test for Equine Piroplasmosis, adds language that will allow horses to be moved on and off the grounds of the racetrack under the original Certificate of Veterinary Inspection (CVI) for 30 days, adds or revises definitions to Horse Medication definitions, sets regulatory limits on medications, and prohibits acts with regard to feed and medication.

The requirement for a negative test for Equine Piroplasmosis is in response to an outbreak of piroplasmosis in Texas and the positive piroplasmosis test of one horse at Canterbury Park in 2010. The one positive test caused the quarantine of an entire barn.

The NSAID portion of definitions has been modified to allow the use of either serum or plasma for medication testing. Currently, serum is used for this process and the rules are being amended to show this. Other definitions are added as they are used elsewhere in the rules or are utilized in the course of the amendments.

The CVI amendment will create parity for all parties, whether in state or out of state, and will reduce the cost for the horsemen and women who take horses on and off the grounds of the racetrack.

The addition of regulatory limits has been a collaborative effort on the part of the Commission and staff, the horsemen and their representative groups to provide better guidance in the administration of therapeutic medications to racing horses.

The use and possession of medicated feed products not intended or labeled for use in horses is added to the prohibited acts. This is done to prevent the introduction of medications that may be harmful to racing horses and for which there is no diagnostic test.

ALTERNATIVE FORMAT

Upon request, this Statement of Need and Reasonableness can be made available in an alternative format, such as large print, Braille, or cassette tape, or digital disc. To make a request, contact Ms. Marlene Swanson at the Minnesota Racing Commission, P. O. Box 630, Shakopee, MN 55379; phone 952-496-7950, fax 952-496-7954; or email at marlene.swanson@state.mn.us. TTY users may call the Racing Commission at 800-627-3529.

STATUTORY AUTHORITY

The Racing Commission's statutory authority to adopt the rules is set forth in Minnesota Statutes section 240.23, which provides: The Commission has the authority, in addition to all other rulemaking authority granted elsewhere in this chapter to promulgate rules governing a) the conduct of horse races held at licensed racetracks in Minnesota, including but not limited to the rules of racing, standards of entry, operation of claiming races, filing and handling of objections, carrying of weights, and declaration of official results, b) wire communications between the premises of a licensed racetrack and

any place outside the premises, c) information on horse races which is sold on the premises of a licensed racetrack, d) liability insurance which it may require of all racetrack licensees, e) auditing of the books and records of a licensee by an auditor employed or appointed by the Commission, f) emergency action plans maintained by licensed racetracks and their periodic review, g) safety, security, and sanitation of stabling facilities at licensed racetracks, h) entry fees and other funds received by a licensee in the course of conducting racing which the Commission determines must be placed in an escrow account, i) affirmative action in employment and contracting by licensed racetracks, and j) any other aspect of horse racing or pari-mutuel betting which in its opinion affects the integrity of racing or the public health, welfare, or safety.

Further statutory rulemaking authority relating to the amendments contained herein include M.S. 240.24, subd. 1, Medication as amended by Laws of 2008, Ch. 318, Article 2, Section 1, and M.S. 240.25, Prohibited Acts.

Under these statutes and session law, the Racing Commission has the necessary statutory authority to adopt the proposed rule amendments.

REGULATORY ANALYSIS

(1) a description of the classes of persons who probably will be affected by the proposed rule, including classes that will bear the costs of the proposed rule and classes that will benefit from the proposed rule

Piroplasmosis is a federally reportable disease contained by disposal of an infected animal and quarantine of the premises. The amendments on Piroplasmosis will benefit all horse owners with horses stabled at one of the two racetracks in Minnesota, all trainers, and racetrack management as infected horses will not be allowed on the grounds.

Owners of racing and non-racing horses shipping in and out of a licensed racetrack will benefit by changing the language so that all Certificates of Veterinary Inspection (CVI) are valid for 30 days after the initial entry to the racetrack.

The amendments to Definitions will benefit horse owners, trainers, and veterinarians as it provides basic information regarding the meaning of words and phrases.

The amendments to Medications and Practices Prohibited provide greater guidance in the use of therapeutic medications in race horses. Class C licenses for race horse owners and trainers will be affected, a positive laboratory test showing any level above the permitted regulatory limit will result in a fine or some other penalty (loss of purse, license suspension) to be determined by the Stewards or Judges. Racetrack management would bear the cost of additional testing required by the proposed rule amendment. The betting public, jockeys, drivers and horses will benefit in that horses will not be racing with performance enhancing medications in their system.

The prohibition of use and possession of medicated feed products not labeled or intended for use in horses keeps these products off the backside of the racetrack and out of the horse's system. Several of these products are poisonous to horses and their misuse has resulted in death. The betting public, jockeys, and drivers, as well as horses, will benefit as several of these medicated feed products are performance enhancing but dangerous and horses will not be racing with any of them in their systems.

(2) the probable costs to the agency and to any other agency of the implementation and enforcement of the proposed rule and any anticipated effect on state revenues

There is no anticipated change in costs to the Commission or to any other state or local agency due to these proposed amendments. The Commission currently tests for these substances, but there will be an increased cost associated with quantification of the amounts. The racetrack management will bear these additional costs.

(3) a determination of whether there are less costly methods or less intrusive methods for achieving the purpose of the proposed rule

Requiring testing for Piroplasmosis is the least costly means of controlling the spread of this disease. The other proposed rule amendments do not change any standard operating procedures in the taking of samples by veterinary staff or testing method that the Commission is currently performing through its testing laboratory. The slight increase to total cost of testing to receive quantitative results will be borne by the racetracks and is the only way to give the horsemen and treating veterinarians the guidance they are requesting.

(4) a description of any alternative methods for achieving the purpose of the proposed rule that were seriously considered by the agency and the reasons why they were rejected in favor of the proposed rule

There are no alternative methods to stop the spread of Piroplasmosis other than disposal of infected animals and quarantining the facility. Screening the horses entering the racetrack facilities is a much more acceptable method of reducing the spread of the disease.

There are no alternative amendments for the certificate of veterinary inspections (CVI). This amendment brings the racetracks in line with the state of Minnesota import requirements for horses.

Definitions are added as housekeeping details with no effect on cost so no alternative amendments were sought.

There are no better alternative methods for the amendments to horse medication. The Commission currently tests for the presence of these substances and will continue to do so. These amendments provide regulatory levels that can be used by horsemen and women and veterinarians when treating horses.

There are no alternative amendments to the prohibited acts. Several of these substances are poisonous to horses and deaths have occurred from their use. The prohibition on the use and possession of medicated feeds not intended for use in horses is the only means of keeping these substances out of a horse's system.

(5) the probable costs of complying with the proposed rule, including the portion of the total costs that will be borne by identifiable categories of affected parties, such as separate classes of governmental units, businesses, or individuals

The cost for testing for Piroplasmosis will be borne by the horse owners. The test is good for a 12 month period and other racetracks, sales venues, and show areas require testing so it will be spread over several jurisdictions, not just Minnesota. No governmental units will be affected.

The cost for a Certificate of Veterinary Inspection is also borne by the horse owners. This is already required by rule. Extending the period that the initial inspection is valid will be a cost savings to those who regularly ship in and out of a racetrack.

Costs for sample collection by veterinary staff and laboratory testing costs for medication violations are currently part of the Commission's budget. These costs are reimbursed by its licensed racetracks. Adoption of the medication amendments will add a slight increase to the total cost of providing these services as medication will need to be quantitated. No governmental units will be affected. Individual horse owners or trainers will be affected only if testing exceeds the regulatory levels contained in these amendments (fines, loss of purse, or license suspension).

There are no expected costs for prohibiting the use and possession of medicated feed products not intended for use in horses.

(6) the probable costs or consequences of not adopting the proposed rule, including those costs or consequences borne by identifiable categories of affected parties, such as separate classes of government units, businesses, or individuals

Not adopting the rule changes for Piroplasmosis testing will cause no change in cost but the consequence may be an infected horse present on the premises resulting in a federal/state quarantine.

There is a disparity under current rule in the way horses residing in state and those shipping in from neighboring states are treated with regard to the Certificate of Veterinary Inspection and also with the rules of the Minnesota Board of Animal Health. Not changing this part of the rule puts a greater financial burden on those owners shipping in and out from neighboring states.

With the proposed amendments to definitions, the Commission desires to provide basic understanding of words and phrases. Not adopting the rule will cause no change in costs but will make it difficult for some racehorse owners and trainers to adequately understand what some particular words and phrases mean.

With the proposed amendments to medications, the Commission desires to provide the horse men and women with guidance regarding the use of these compounds. Not adopting the rule will result in less cost but the consequence may increase medication violations and confusion regarding medication administration.

Not adopting the proposed amendment prohibiting the use and possession of medicated feed products not specifically labeled for use in horses will cause no change in cost. The consequence, however, may be illness and death in a racehorse as well as illegal use of medications which may have a performance enhancing effect.

(7) an assessment of any differences between the proposed rule and existing federal regulations and a specific analysis of the need for and reasonableness of each difference

Piroplasmosis is a federally reportable disease with testing currently only required for horse import. In recent years, however, the disease has become more widespread at racetracks where large groups of horses are comingled and moved around the country with great regularity. There is a need to protect these groups of horses and yet not penalize those horses in Minnesota that are not exposed to the infectious agent nor move around the country.

The generation of Health Certificates (CVI's) is governed by the USDA, but the state import regulations are governed by state rule and/or statute. The proposed rule amendment to allow all horses, regardless of state of origin, to use the CVI for 30 days would be similar to Minnesota rule and statute for importation of horses into Minnesota.

To the best of our knowledge, with regard to the definition changes, there are no differences between these proposed rule amendments and current federal requirements.

There are no current federal rules regarding the establishment of medication regulatory limits.

There are no current federal rules regarding the prohibition of the use and possession of medicated feed products not intended for use in horses.

PERFORMANCE-BASED RULES

The Commission's mission statement states, "The Minnesota Racing Commission was established to regulate horse racing and card playing in Minnesota to ensure that it is conducted in the public interest, and to take all necessary steps in ensuring the integrity of racing and card playing in Minnesota thus promoting the breeding of race horses in order to stimulate agriculture and rural agribusiness." These proposed rule amendments affect horse racing and are being proposed as means to strengthen the Commission's statutory authorized regulatory oversight so as to ensure the continued integrity of this form of legalized gambling. Any actual occurrence or even the perception that the integrity has been compromised would have a disastrous effect on not only the racetracks but also those that compete at the racetracks, many of whom rely on this activity for their livelihood. In proposing rule amendments, not only in this case but in all others as well, the Commission and its staff, constantly strive to be aware of ways by which the integrity of racing and pari-mutuel wagering can be improved and strengthened while at the same time proposing rules that allow flexibility by racing participants and Commission staff in responding to unanticipated situations in a business like fashion. This is done during the conduct of regulatory duties and responsibilities on a day to day basis and by staying current on national issues, especially medication issues, with regard to these proposed amendments.

ADDITIONAL NOTICE

These proposed amendments were discussed at regularly scheduled Commission meetings and Commission Work Sessions. All rules discussion was clearly included on all agenda duly prepared and mailed or e-mailed 7 days prior to these meetings. Agendas were also posted on the Commission's website. The meetings were held on August 24, September 2, September 7, October 5, October 21, November 1, November 18, December 7, and December 16 of 2010. The rules setting regulatory limits were also discussed at the meetings on January 4, January 20, February 17, March 1, and March 17 of 2011. Minutes from the full commission meetings are available on the Commission's website at www.mrc.state.mn.us.

The Racing Commission began work on the rules proposals in August of 2010 and has provided updates on the status of the rulemaking proceedings at its monthly meetings. Continued updates were provided as needed during the course of the formal rulemaking process.

The Commission's Rulemaking Docket, which is publicly posted in the Commission's office as well as on the Commission's website, will be updated as necessary to reflect the status of these rules.

In addition to the discussions at the Racing Commission work sessions and full commission meetings, a committee was formed for the development of the rules setting regulatory limits for therapeutic medications. The Committee consisted of the following individuals:

- Linda Arnoldi, Director of Pari-Mutuel Operations, Canterbury Park, and former horse owner
- Kelvin Childers, Board Member, Minnesota Quarter Horse Racing Association
- Dr. Lynn Hovda, DVM, Chief Veterinarian for the Minnesota Racing Commission
- Kay King, Executive Director, Minnesota Thoroughbred Association
- Dr. Christy Klatt, DVM, Assistant Commission Veterinarian, Minnesota Racing Commission
- Richard Krueger, Executive Director, Minnesota Racing Commission
- Dr. Sandy Larson, DVM, Private Veterinarian, Canterbury Park
- Mary Manney, Deputy Director, Minnesota Racing Commission
- Dr. Rod Moberg, DVM, Private Veterinarian, Running Aces Harness Park
- Dr. Thomas Tobin, University of Kentucky and consultant to Minnesota Horsemen's Benevolent & Protective Association
- Patrice Underwood, Executive Director, Minnesota Horsemen's Benevolent & Protective Association
- Jack Walsh, Vice President, Minnesota Horsemen's Benevolent & Protective Association
- Dr. Scot Waterman, Executive Director, Racing Medication and Testing Consortium

The meetings of this committee included phone consultations with Dr. Scott Waterman, Executive Director of Racing Medication Testing Consortium (RMTC), and Dr. Thomas Tobin, an expert and author of Equine Drug Testing and Therapeutic Medication.

Our Notice Plan includes:

1. Publishing the Request for Comments in the November 21, 2011 edition of the State Register.
2. Posting the Request for Comments and the language of the proposed rules on the Commission's website.
3. Mailing or e-mailing the Request for Comments to Class A & B licensees as well as horsemen's organizations that are affected by horse racing in Minnesota, including the Minnesota Thoroughbred Association, the Horsemen's Benevolent and Protective Association, Minnesota Harness Racing, Inc., the Minnesota Quarter Horse Racing Association, the Jockey's Guild, and the United States Trotting Association.
4. Mailing or e-mailing the Request for Comments to organizations in Minnesota identified as having an interest in animal health including the Minnesota Board of Animal Health, the Minnesota Humane Society, the Minnesota Veterinary Medical Association, and the University Of Minnesota College Of Veterinary Medicine.
5. Our Notice Plan also includes giving notice required by statute. We will mail the rules and Notice of Intent to Adopt to everyone who has registered to be on the Commission's rulemaking list under Minnesota Statutes, section 14.14, subdivision 1a. We will also give notice to the Legislature per Minnesota Statutes, section 14.116. The Proposed Rules and the Notice of Intent to Adopt will also be published in the State Register.
6. The Commission will provide a copy of the rules and Notice of Intent to Adopt Rules to Class A & B licensees, horsemen's organizations, and animal health organizations in Minnesota as noted in #3 and #4.

CONSULT WITH MMB ON LOCAL GOVERNMENT IMPACT

As required by Minnesota Statutes, section 14.131, the Department will consult with the Minnesota Management and Budget (MMB)). We will do this by sending the MMB copies of the documents that we

send to the Governor's Office for review and approval on the same day we send them to the Governor's office. We will do this before the Commission's publishing the Notice of Intent to Adopt. The documents will include: the Governor's Office Proposed Rule and SONAR Form; the proposed rules; and the SONAR. The Department will submit a copy of the cover correspondence and any response received from Minnesota Management and Budget to OAH at the hearing or with the documents it submits for ALJ review.

DETERMINATION ABOUT RULES REQUIRING LOCAL IMPLEMENTATION

As required by Minnesota Statutes, section 14.128, subdivision 1, the agency has considered whether these proposed rules will require a local government to adopt or amend any ordinance or other regulation in order to comply with these rules. The Commission has determined that they do not because all activity that these amendments affect occur on licensed racetrack grounds, not out in the local community. There are times where we may have to contact local law enforcement or county/city attorney offices, but that is in the normal course of fulfilling our duties and responsibilities when events warrant. It is not anticipated that these amendments will either increase or decrease those contacts.

COST OF COMPLYING FOR SMALL BUSINESS OR CITY

Agency Determination of Cost

As required by Minnesota Statutes, section 14.127, the Racing Commission has considered whether the cost of complying with the proposed rules in the first year after the rules take effect will exceed \$25,000 for any small business or small city. The Racing Commission has determined that the cost of complying with the proposed rules in the first year after the rules take effect will not exceed \$25,000 for any small business or small city.

LIST OF WITNESSES

If these rules go to a public hearing, the Racing Commission anticipates having the following witnesses testify in support of the need for and reasonableness of the rules:

1. Mr. Richard G. Krueger, Executive Director of the Commission will testify about the development and content of the rules.
2. Ms. Mary Manney, Deputy Executive Director of the Commission will testify about the development and content of the rules.
3. Dr. Lynn Hovda, Chief Veterinarian of the Commission will testify about the development and content of the medication and testing proposals.
4. Ms. Marlene Swanson, Rules Coordinator will testify about the development and processing of these rules.

RULE ANALYSIS

Minnesota Statutes, chapter 14, requires the Commission to explain why a rule amendment is needed and why the amendment is the correct choice. The following analysis will explain why each amendment is needed and why it is a reasonable response with a rational basis. The need for and reasonableness of the proposed rules, amending Minnesota Rules parts 7876.0100, 7876.0110, 7876.0120, 7890.0100, 7890.0110, and 7897.0100 is as follows.

Minn. Rules, part 7876.0100 On Track Stabling, 7876.0110 Off Track Stabling, and 7876.0120 On and Off Track Stabling of Non-racing Horses.

The amendments to 7876.0100, 7876.0110, and 7876.0120 add the same language to all three parts and do two things. The first part of the amendment to these rules modifies the language regarding timing and original health certificates in 7876.0100, subp.10, 7876.0110, subp.4, and 7876.0120. The second part adds language requiring a negative test for Equine Piroplasmosis in 7876.0100, subp.11, 7876.0110, subp.5, and 7876.0120.

The amendment to 7876.0110, subp.4, strikes the language allowing trainers with horses shipping in and out with health certificates originating in Minnesota to use this same certificate for 30 days. The new language added to those parts dealing with original health certificates and non racing horses extends this 30 day usage to all health certificates and all horses shipping in and out.

Need for the amendments regarding time:

Whether horses are stabled at a racetrack or at an off- site facility it is not unusual for them to be shipped into the grounds of a Minnesota Racing Commission (MRC) licensed racetrack. Many horses ship in from another racetrack or from an outside stabling facility for a race. Some horses thrive far better on pasture and ship in daily for training and/or racing and return to their home facility after each event. There are also many non-racing horses, used to accompany racehorses as they warm up on the track and to take the racehorses to the starting gate, that return to their own off track barns or stables at the end of each day and/or event. These horses ship in and out on a daily or weekly basis depending on the needs of trainers and race days. Under current MRC rules a health certificate also known as a certificate of veterinary inspections (CVI) needs to be issued 10 days prior to entry to the racetrack and are valid for 30 days *only for those horses residing in the state of Minnesota* and not those shipping in from other states such as Wisconsin, Iowa, Illinois, South Dakota, and others. This penalizes horsemen and women in neighboring states by requiring a new CVI every 10 days and is dissimilar to the Minnesota Board of Animal Health rules.

Justification for the amendments regarding time:

This rule change would allow all horses, regardless of their state of origin, to utilize a CVI for a full 30 days from the day of issue. Not only would the horsemen and women save a considerable amount of money but it would be easier and more convenient for them to ship their horses into an MRC licensed racetrack. In addition, the Minnesota Board of Animal Health rules for importation of horses state that the CVI is good for 30 days prior to importation¹. This disconnect between MRC rules and Board of Animal Health rules is confusing to horsemen and women in other states and the MRC seeks to clarify this by making CVIs valid for 30 days from issue once the horse has entered the grounds of an MRC racetrack for the first time.

No change is requested regarding the need for the certificate to be issued 10 days prior to entry. The MRC is more restrictive than the Board of Animal Health for first entry into the racetrack for a number of reasons. Horses come from many parts of the country into a very tight, closed environment and accidental entry of infectious diseases needs to be prevented. The short 10 day window of time ensures that owners and trainers ship in horses that are healthy and do not have any known infectious diseases. Once the horses have arrived and are deemed healthy by the trainer and their personal veterinarian they are free to ship in and out as needed. In addition, the short time period allows both the trainer's veterinarian and MRC regulatory veterinarian to become familiar with any known issues a horse may have and effectively communicate any suspected issues with regulatory veterinarians at the originating

racetrack. Finally, there is a discrepancy between date of inspection and date of issue (up to 10 days in Minnesota) which may lengthen the time period considerably².

Need for the amendments regarding Equine Piroplasmiasis:

Minn. Rules 7876.0100, subp.10, 7876.0110, subp.4, and 7876.0120 already require any horse entering the grounds of a racetrack licensed by the Minnesota Racing Commission (MRC) to have a certificate of veterinary inspection (CVI or “health certificate”) issued not more than 10 days prior to arrival at the race track. The CVI must be issued by a USDA accredited veterinarian and must contain complete equine infectious anemia (EIA) test results. The proposed rules add to this requirement an Equine Piroplasmiasis (EP) test taken within 12 months of the arrival at the racetrack, with negative results for *Theileria (Babesia) equi* and *Babesia caballi*.

This rule is needed to allow the MRC to rapidly respond to any active cases of EP in North America and protect owners and trainers as well as all horses stabled or shipping into MRC licensed racetracks from exposure to EP. In 2010, routine EP testing required by another racetrack for entry showed a positive horse that was stabled at Canterbury Park. The barn where the horse was stabled and 32 horses stabled in that barn were quarantined by the USDA Area Veterinarian in Charge (VIC) and the Minnesota Board of Animal Health³ for a minimum of 30 days until further testing showed no positive horses. In addition, several other horses on the farm of origination were tested and quarantined with another positive horse identified. Both positive horses were euthanized by the Minnesota Board of Animal Health Area Veterinarian in Charge as there are currently no effective treatment options recognized by the USDA for horses in the United States and complete isolation was not available^{4,5}.

Justification for the amendments regarding Equine Piroplasmiasis:

Equine Piroplasmiasis is a federally reportable disease not endemic to the United States. The federal government currently only requires EP testing for horse imports; they do, however, require reporting of the disease if found in any horses within the United States. Equine piroplasmiasis, a blood parasite, is a tick borne disease and the ticks carrying the parasites can be moved by animals, human beings, trailers, bedding, feed, and other vegetation. Similar to equine infectious anemia, it can also be spread by the use of blood contaminated needles, syringes, and surgical equipment. In recent years the disease has become more widespread with sporadic cases occurring at training facilities and racetracks where large numbers of horses are commingled and moved around the country with ease and regularity^{5,6}. Once an infected horse is identified, the disease is contained by complete isolation or quarantine of the premises and disposal of the infected horse. The USDA does not recognize any medical options used in foreign countries to treat the disease. These treatments may cure the illness but not kill the parasite in the blood leaving a chronic source of infection⁷. Requiring EP testing prior to entry from horses originating in area with known active cases is justified as it will prevent future quarantines, protect equine athletes currently residing at MRC licensed racetracks from illness and death, and save owners from devastating economic and emotional losses.

Minn. Rules, part 7890.0100 Definitions.

The proposed amendments to 7890.0100 adds definitions for 7890.0100, subp.11a, Intra-articular(IA)⁸, 7890.0100, subp.11b, Intramuscular(IM)⁹, 7890.0100, Subp.11c, Intravenous(IV)¹⁰, and 7890.0100, subp.14c, Orally(PO)¹¹ which define the route of administration or the means by which the drug is given¹². The additional definitions to 7890.100, subp.12a, Limit of Detection (LOD)¹³, 7890.0100, subp.12b, Limit of Quantitation(LOQ)¹⁴, and 7890.0100, subp.15a, Regulatory Limit¹⁵, help with understanding laboratory analysis of medication testing.

Need for amendments regarding definitions:

The first four additions above refer to means or methods of administering a medication. The addition of these definitions is needed so all parties involved in racing clearly understand what is meant when referring to medication administration and what each abbreviation refers to.

The last three definitions refer to the laboratory analysis portion of medication testing and need to be added so owners, trainers, veterinarians, and regulators are familiar with them. They are often used when discussing specific medications and medication testing and thus the need to know what they mean is important for anyone involved in the horse racing industry.

All of these definitions are also needed because these terms may be referred to in other areas of the rules and have not yet been clearly defined in the rules.

Justification for the amendments regarding definitions:

It is important that the specific language used for medication administration is understood. These terms are not necessarily common to all parties involved and adding the definitions makes it clear what these terms mean. The definitions for terms regarding laboratory analysis are justified as they are used by owners, trainers, veterinarians, and regulators to describe the laboratory reporting of medication testing. These reporting definitions were provided to the Minnesota Racing Commission (MRC) by Dr. Scot Waterman, executive director of the Racing Medication and Testing Consortium (RMTTC), and found in a 2010 PowerPoint presentation given to Vision 20-20, a Thoroughbred racehorse leadership group. The RMTTC is the organization involved with equine medication testing, research, and the establishment of regulatory limits for many common medications used in a therapeutic manner by racehorses. This organization strives to develop and promote uniform rules, policies and testing standards at the national level. For easy reference and additional information, Dr. Waterman's complete PowerPoint presentation is provided for the reader as Exhibit 9 in this SONAR.

Minn. Rules, part 7890.0100 Definitions. Subp.13 Medication

The amendment to 7890.0100, subp.13, item A, in general does more than define a term as the previously discussed amendments to definitions do. This amendment adds guidance to the use of Non-Steroidal Anti-Inflammatory Drugs (NSAIDS).

Need for amendments regarding NSAID definitions:

The Minnesota Racing Commission (MRC) attempts to operate under the model rules set forth by the Association of Racing Commissioners International (ARCI, <http://www.arci.com>). This national organization provides guidance and uniform model rules to state racing commissions so horsemen and women can easily travel from state to state and work under similar sets of circumstances. These proposed amendments conform to the ARCI manual and need to be adopted so that horsemen and women can follow the same non-steroidal anti-inflammatory drug rules as they move from one racing jurisdiction to another.

Justification for the amendments regarding NSAID definitions:

The amendments to 7890.0100, subp.13, item A, subitem (1), subp.13, item A, subitem (2), subp.13, item A, subitem (3), and subp.13A, item A, subitem (4) conform to the current ARCI model rules for non-steroidal anti-inflammatory drugs as found in Chapter 11 of the ARCI document. (ARCI-011-020, Medications and Prohibited Substances, E1a, E1b, E1c, and E2)¹⁶. The adoption of these rules is justified as it prevents accidental drug overages due to non conforming rules, keeps administered medications and the timing thereof consistent with national standards, and prevents unintentional medication administration errors.

Minn. Rules, part 7890.0100 Definitions. Subp.13 Medication, Item A, subitems (1) a, b, and c

In addition to expanding the NSAID definition to conform to ARCI rules, the terms "of the substance" and "or serum" have been added to the existing rules in 7890.0100, subp.13, item A, subitems (1) a, b, and c.

Need for amendment regarding the words “of the substance” in 7890.0100, subp.13, Item A, subitems (1) b and c:

Addition of the words “of the substance” in subitems b and c is needed to promote consistency through the subpart and the rules. In other words, all three subitems (a, b, and c) will have similar language. This makes it easier for anyone searching for and reading the rules to understand them more clearly.

Justification for amendment regarding the words “of the substance” in 7890.0100, subp.13, Item A, subitem (1) b and c:

This is justified as it makes the rules uniform and consistent.

Need for amendment regarding the words “or serum” in 7890.0100, subp.13, Item A, subitems (1) a, b and c:

The Minnesota Racing Commission (MRC) attempts to operate under the model rules set forth by the Association of Racing Commissioners International (ARCI; www.arci.com). This organization provides guidance to state racing commissions and suggests rules so that horsemen and women can easily travel from state to state and work under the same set of rules without penalty. Current ARCI model rules for non-steroidal anti-inflammatory drug testing recommend the use of either plasma or serum as the testing medium and this rule change is needed to bring the MRC into line with the ARCI.¹⁶ From a drug testing standpoint, there is little difference between the two and both provide similar results.¹⁷ The choice, however, does allow racing commissions to choose a sample that best fits their current testing abilities and requirements, considers input from their contracted testing laboratory, and makes it easy for laboratories across the country operating under the model rules to accept split sample requests from horsemen and women.

Justification for amendment regarding the words “or serum” in 7890.0100, subp.13, Item A, subitems (1) a, b and c:

Typically whole blood is collected from horses chosen by the Stewards for testing and processed either as plasma or serum. The choice is made at the time of collection when an appropriate tube is chosen.^{18, 19} After blood is collected, it is processed in a similar manner using a piece of laboratory equipment called a high speed centrifuge that rapidly spins them and separates the blood and fluid components.¹⁹

- *Plasma tubes* contain an anticoagulant such as sodium or lithium heparin which prevents the blood from clotting.¹⁷ They can be spun down immediately without sitting. When spun down at high speeds (centrifuged) the fluid (plasma) portion contains platelets and coagulation factors and the cellular portion contains red and white blood cells.¹⁸ A firm clot is not formed due to the presence of the anticoagulant and shaking or agitating the tubes often “remixes” the substances.
- *Serum tubes* contain no anticoagulant and essentially provide a more purified version of plasma. They must sit for at least 30 minutes prior to being spun down.

When spun down at high speeds (centrifuged), they separate into a firm clot containing red and white blood cells as well as platelets and coagulation factors.¹⁸ A softer fibrin clot is generally formed as well. The remaining fluid, referred to as serum, sits on the top of the clotted portion and once spun down is stable and does not “remix” if agitated.

The figure below (taken from Exhibit 11) is included to show the difference between whole blood, plasma, and serum.

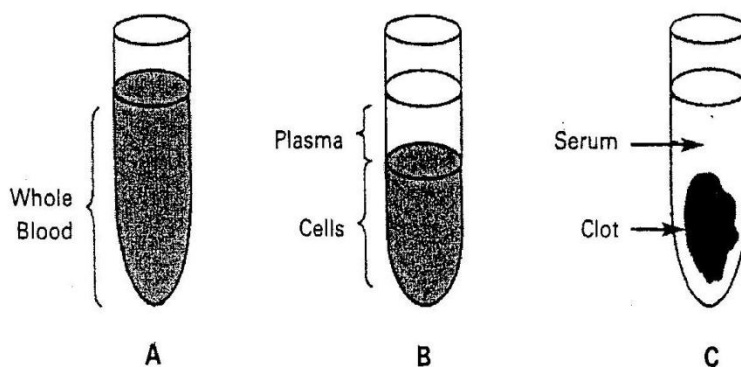


Fig. 2.2. Physical difference between plasma and serum. (A) Mixed fluid and cells in whole blood. (B) With anticoagulant added, cells separate from plasma. (C) Without an anticoagulant, cells and clotting factors separate from serum.

There are advantages and disadvantages to both processes. When time is important such as in human hospitals or when the sample will not be subjected to agitation and remixing from transport by vehicle or airplane, plasma is an acceptable choice.^{17, 20} If time is not important or if the sample needs to be stored for a longer period of time and transported long distances, serum is a more acceptable choice.^{17, 20} When the original rules were written the MRC utilized a local testing laboratory (University of Minnesota Veterinary Diagnostic Laboratory, UMVDL) and samples needed only to be transported across town on a daily basis. The UMVDL no longer supports a drug testing program and the MRC now utilizes a private contract laboratory (currently Industrial Laboratories, Denver, CO). Samples are stored for several days and shipped either by airplane or FedEx to the testing laboratory.

Minnesota Statute, section 240.24, as written in 1983, gave the Racing Commission the authority to make and enforce rules governing medication and medical testing for horses running at licensed racetracks. The statute gave the commission considerable latitude to define the terminology and procedures to accomplish this directive. Minnesota Statute, section 240.24, subdivision 2, the basis for this rule amendment, was added to section 240.24 in 1985, and amended in 2001 changing the level of micrograms.²¹ Though the statute is specific in stating “does not contain more than five micrograms of the substance of metabolites thereof per milliliter of blood plasma”, it still allows the commission latitude when it defines test sample as “any bodily substance including blood, urine, saliva, or other substance as directed by the commission”. At that time, the University of Minnesota was conducting the lab tests and plasma was an appropriate medium to use for testing. Since 1985 technology has changed and there are no local labs to do the testing. Amending the rule by adding the words “or serum” and striking some existing language makes the rule more readable and consistent with subitems b and c. It is within the scope of the commission’s authority and is consistent with the intent of the legislature.

Minn. Rules, part 7890.0110 Medications and Practices Prohibited, Subp.5 Presence.

The proposed change to *Minn. Rules, part 7890.0110, subp.5* makes the language in this subpart identical to the language in 7890.0100, subp.13, item A, subitem 3.

Need for amendment regarding 7890.0110, subp.5:

The need for this language is explained under the need and justification regarding NSAID definitions on page 14 of this document. The need for repeating this language in 7890.0100, subp.5 is the importance of this particular rule and that it will be seen by the reader whether they access “definitions” or “medications and prohibited acts”.

Justification for amendment regarding 7890.0110, subp.5:

This rule is justified as it provides accurate information regarding the use of non steroidal anti-inflammatory drugs (NSAIDS) in Minnesota race horses and provides instruction on when other medications need to be discontinued. It is justified to repeat this language to avoid the misuse of NSAIDS.

Minn. Rules, part 7890.0110 Medications and Practices Prohibited, Subp.10 Medications with regulatory limits.

“Subp.10 Medications with regulatory limits. No medications other than those listed below or found in 7890.0100, subp. 13 A through D, shall be allowed in the test sample of a horse. Levels on the following medications shall not exceed:” and then a list of specific drugs and regulatory limits are added to part 7890.0110 Medications and Practices Prohibited to provide guidance in the administration of therapeutic medications.

Need for amendment regarding 7890.0110, subp.10:

This rule is needed to provide trainers and private veterinarians working at the racetrack guidance regarding the use of common therapeutic medications and help prevent inadvertent medication violations for trace amounts of medications that have no effect on racing performance. Medication testing has become increasingly more sophisticated and medications are being found in serum and urine in microscopic amounts that have no effect on racing performance. The proposed amendments do not change any standard operating procedures in the taking of samples by veterinary staff or testing methods that the Minnesota Racing Commission (MRC) is currently performing through its testing laboratory. They simply provide guidance to veterinarians and horsemen and women for the safe administration of several common therapeutic medications.

Justification for amendment regarding 7890.0110, subp.10:

A statute change in 2008, (Laws of 2008, Chapter 18, Article 2, Section 1) allowed the MRC to establish regulatory limits for medications. This was necessary as laboratory testing for medications has become very sophisticated, often finding miniscule amounts of medications that have no effect on racing performance. When the rules and statutes were originally written, medications were reported in parts per million. Currently, medications are reported in parts per trillion or lower. To put this in perspective, one part per trillion is equivalent to one second in 32,000 years.²²

A working group was established in November 2010 by the Chair of the MRC to develop regulatory limits for the common therapeutic medications. The group consisted of representatives from the MRC, Canterbury Park, breed groups, horsemen, private veterinarians working the backside at both Running Aces and Canterbury Park, and regulatory veterinarians working for the MRC.²³ Dr. Scot Waterman, Executive Director for the Racing Medication and Testing Consortium (RMTTC), provided guidance.²³ Medications were proposed by individuals and the original list of 23 medications was eventually narrowed to fifteen. In order to be included on the final list, each medication needed to be found in a study with results pending or reported by the RMTTC, published as an international standard in equine racing, supported by published scientific research, or be part of a study performed by a licensed and

accredited diagnostic and testing laboratory with the intent to be published. Dr. Scot Waterman provided a wealth of information based on RMTC studies. In 2007, the RMTC assembled a list of 50 commonly used therapeutic medications identified by horsemen and women in many jurisdictions and is working towards establishing regulatory limits for each medication.^{24, 25} To achieve this goal, the RMTC supports a herd of 20 research horses at the University of Florida specifically used for drug administration testing.²⁶ When drug administration results and statistical analysis are completed results are provided to the ARCI (www.arci.com) for inclusion in model rules.

The fourteen proposed medications and the corresponding regulatory limits, with the supporting documentation as footnotes, are as follows:

Drug	Regulatory Limit
1. 2-(1-hydroxyethyl)promazine	10 ng/ml (U) ^{27 28}
2. Clenbuterol	25 pg/ml (S or P) ^{29 30 31}
3. Dantrolene or hydroxy-dantrolene	50 ng/ml (U) ^{32 33}
3a. Dantrolene or hydroxy-dantrolene	0.1 ng/ml (S or P) ^{32 33}
4. Detomidine or metabolite(s)	10 ng/ml (U) ^{34 35}
5. Dexamethasone	0.1 ng/ml (S or P) ^{31 36}
6. Diclofenac	0.2 ng/ml (S or P) ^{37 38 39}
7. DMSO	10 mcg/ml (S or P) ⁴⁰
8. Firocoxib	40 ng/ml (S or P) ⁴⁰
9. Glycopyrrolate	2.0 pg/ml (S or P) ^{40 41 42}
10. Isoxuprine	50 ng/ml (S or P) after screening level in urine > 500 ng/m ^{43 44}
11. Methocarbamol	1 ng/ml (S or P) ^{31 40 45}
12. Methylprednisolone	0.1 ng/ml (S or P) ^{31 36}
13. Pyrilamine	50 ng/ml (U) ^{27 35}
14. Triamcinolone Acetonide (Vetalog®)	0.1 ng/ml (S or P) ^{31 36}

Minn. Rules, part 7897.0100 Prohibited Acts, Subp.20 Possession or use of a drug, substance, medication, biological product, or venom

Language has been added as 7897.0100, subp.20 D, *No person shall feed, or cause or knowingly permit to be fed or connive in any manner to feed products that contain any drug not permitted by chapter 7890 to a horse to be entered or entered for a race.*

Need for amendment regarding 7897.0100, Subp.20 D:

Anabolic steroid drugs are now tightly controlled at equine racetracks and these controls have effectively eliminated their use.⁴⁶ Unscrupulous trainers have looked elsewhere to find products that will make their horses grow bigger and taller, develop more muscle mass, and run faster.^{46 47 48} Medications or substances added to feeds to promote acceptable market growth in pigs, cattle, and lambs⁴⁹ have a high potential for abuse in race horses. These products are not labeled for use in horses and indiscriminate use has resulted in illness and death.^{46, 50 51} This rule is needed as these products have no place in the body system of horses entered or to be entered to race. These are performance enhancing drugs that adversely affect the health and well being of race horses.⁴⁶

Justification for the amendments regarding 7897.0100, Subp.20 D:

It took the MRC several years and a statute change to control the use of anabolic steroids in horses racing in Minnesota. Prior to that time, it was not uncommon for horses to receive not one but two or three anabolic steroids as frequently as once a month.⁵² Other products not labeled for use in horses have been used with a mechanism of action similar to anabolic steroids, in particular medicated feed products such as ractopamine and zilpaterol, in an attempt to reproduce the effects seen with anabolic steroids.^{46 47 48} Some feed products contain both an anabolic steroid like product and other medications, such as monensin.^{50 51} Monensin is highly toxic to horses even in small amounts and ingestion often results in death.⁵⁰ This proposed rule is justified as it will prevent trainers from circumventing the anabolic steroid rule by feeding these products to their horses, protects the valuable investment of the owner, and keeps horses safe from deliberate or unintentional poisoning.

Language has been added as 7897.0100, subp.20 E, *No person shall have in his possession, within the confines of a racetrack or within its stables, buildings, sheds or grounds, where horses are lodged or kept which are eligible to race over a racetrack, any feed products containing drugs not otherwise expressly approved for use in race horses.*

Need for amendment regarding 7897.0100, Subp.20 E:

This rule change is needed to protect the integrity of horse racing in Minnesota as well as the health of racehorses. This proposed rule is an extension of subpart 20D and prevents trainers from having feed products containing drugs in their possession that are not specifically approved for use in horses. As stated in subpart 20 D, most of these medicated feeds are labeled specifically for use in food producing animals such as swine, cattle, and lambs^{49 50 51} and even at low doses, result in deleterious effects in horses.^{48 50 51} They are also performance enhancing drugs that may have an effect on the outcome of a race. Some are easy to find in the urine and others more difficult or not yet tested for on a routine basis.^{47 53} Their very presence, however, in a trainer's tack room indicates intent to circumvent the rules and feed them to horses as there are no pigs, lambs, or cattle anywhere on the backside of a Minnesota licensed racetrack.

Justification for the amendments regarding 7897.0100, Subp.20 E:

These types of medicated feed products have no place on the backside of a racetrack or in any trainer's tack room. They are not meant to be used in horses, race or otherwise, and their very presence indicates a willingness to not only break the rules but abuse horses. This proposed rule is justified as it takes these products out of the hands of unscrupulous trainers looking for an unfair advantage, keeps drugs that cannot currently be found in urine or serum or plasma by routine laboratory testing off the backside, prevents their use in race horses, and may ultimately save a horse's life. In addition to protecting the horse, this rule ultimately protects trainers and owners as finding these medications in the urine or serum is a serious violation generally met with a fine and prolonged suspension (\$10,000 and one year suspension for first penalty).⁴⁶

CONCLUSION

Based on the foregoing, the proposed rules are both needed and reasonable.

Date

Richard G. Krueger
Executive Director

¹ [Minn. Rules, part 1700.2200](#), Certificate of Veterinary Inspection for Horses

² Exhibit 1. Minnesota Certificate of Veterinary Inspection detailed sample certificate. Provided in a letter to all accredited veterinarians from Minnesota Board of Animal Health, December 14, 2010.

³ Exhibit 2. Linda C. Glazer, DVM DACVPM, Board of Animal Health

⁴ Exhibit 3. Racing Commissioners International (RCI), President's Report: June, 2010

⁵ Exhibit 4: Knowles D. Understanding Piroplasmiasis. *Equine Disease Quarterly* 2010; 19(1): 4-5; http://www.ca.uky.edu/gluck/q_jul10.asp#piro

⁶ Exhibit 5: APHIS Veterinary Services Info Sheet. Protect your horses from equine piroplasmiasis. United States Department of Agriculture. April 2010. http://www.aphis.usda.gov/animal_health/animal_diseases/piroplasmiasis/downloads/ep_protect_your_horses_en_sp.pdf

⁷ Exhibit 6: APHIS Veterinary Services Info Sheet; and

Exhibit 7: Traub-Dargatzis J, Bischoff B, James A, et.al. A literature review of equine piroplasmiasis. United States Department of Agriculture. http://www.aphis.usda.gov/animal_health/animal_diseases/piroplasmiasis/downloads/ep_literature_review_september_2010.pdf

⁸ Merriam-Webster free dictionary. <http://www.merriam-webster.com/dictionary/intra-articular>

⁹ Merriam-Webster free dictionary, <http://www.merriam-webster.com/dictionary/intramuscular>

¹⁰ Merriam-Webster free dictionary, <http://www.merriam-webster.com/dictionary/intravenous>

¹¹ MedicineNet.com, <http://www.medterms.com/script/main/art.asp?articlekey=8310>

¹² Exhibit 8: Bill, RL. Pharmacokinetics and Pharmacodynamics: The Principles of How Drugs Work. *In: Clinical Pharmacology and Therapeutics for the Veterinary Technician*, ed 3. St. Louis MO, 2006, pp 49-51.

¹³ Exhibit 9: Waterman S. Drug Control in Horse Racing in the U.S. Slide #5 Available at: www.rmtcnet.com/resources/RMTC_Vision_20-20_March_2010.ppt

¹⁴ Exhibit 9: Waterman S. Drug Control in Horse Racing in the U.S. Slide #5

¹⁵ Exhibit 9: Waterman S. Drug Control in Horse Racing in the U.S. Slide #7

¹⁶ ARCI Model Rules, Chapter 11, Equine Veterinary Practices, Health and Medication. ARCI-011-020 Medications and Prohibited Substances, E. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). October 2011 <http://www.ua-rtip.org/industry/modelrules_pdfs/chapters5_11.pdf>

¹⁷ Exhibit 10: Uges, DR. Plasma or serum in therapeutic drug monitoring and clinical toxicology. *Pharm World Sci* 1988; 10(5): 185-188.

¹⁸ Exhibit 11: Voigt GL. Blood Composition. *In: Hematology Techniques and Concepts for Veterinary Technicians*, ed 1. Ames, IA: 2000, pp7-12.

¹⁹ Exhibit 12: Thrall MA. Sample collection, processing, and analysis of laboratory service options. *In: Veterinary Hematology and Clinical Chemistry*, ed 1. Philadelphia, PA: Lippincott, 2004, pp39-44.

²⁰ Exhibit 13: Chapter 2.4.1. Whole Blood, plasma, or serum? *In: The Clinical Chemistry – Point of Care*. Available at: www.diagnosticsample.com/point-of-care/introduction.php. Accessed October 13, 2011.

²¹ <http://www.revisor.mn.gov/laws/?doctype=Chapter&year=2001&type=0&id=59>

²² Exhibit 14: Tobin T. Benzoylcegonine thresholds in horse urine. *In: Equine Disease Quarterly* 2007; 16(3): 5-6. Available at: www.ca.uky.edu/gluck/q_jul07.asp. Accessed October 17, 2011.

²³ Exhibit 15: Members of Regulatory Limits Working Group, established November 2011.

²⁴ Exhibit 16: RMTCL list of priority medications, provided by Dr. Scot Waterman to MRC commissioners. October 21, 2010 MRC full commission meeting.

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- ²⁵ Exhibit 17: Waterman S. Medication: Past, present, and future in racing and performance horses. Powerpoint Presentation. Monday, December 8, 2008. San Diego, CA. Available at: www.aaep.org/images/files/ScientificPrgm. Accessed: October 16, 2011.
- ²⁶ Exhibit 18: Waterman S. RMTC Pharmacokinetics and drug withdrawal studies. Powerpoint presentation given to MRC commission. October 21, 2010.
- ²⁷ Exhibit 19: RMTC News Release. October 1, 2009. Available at: www.rmtcnet.net. Accessed: October 15, 2011.
- ²⁸ Exhibit 20: Personal Communication with Dr. Rick Sams, Lab Director, HFL Sport Science. HFL Sport Science has nearly 50 years of continuous experience in the science of sports doping control (equine, canine, human) and helped shape the Rules and Regulations of Racing. http://www.lgc.co.uk/divisions/hfl/hfl_sport_science.aspx
- ²⁹ Exhibit 21: Soma LR, Uboh CE, Guan F, et al. Pharmacokinetics and disposition of clenbuterol in the horse. *J Vet Pharmacol Ther* 2004; 27(2):71-77.
- ³⁰ Exhibit 22: California takes its drug testing seriously. Available at: www.ntra.com/content/safetyalliance/view/NDA. Accessed October 18, 2011.
- ³¹ Exhibit 23: Personal Communication based on RMTC studies. Dr. Scot Waterman, August 30, 2010.
- ³² Exhibit 24: Knych HK, Arthur RM, Taylor A, et al. Pharmacokinetics and metabolism of dantrolene in horses. *J Vet Pharmacol Ther* 2011; 34(3): 238-246.
- ³³ Exhibit 25: Personal Communication, Heather Knych, December 9, 2010.
- ³⁴ Exhibit 26: Grimsrud KN, Mama KR, Thomasy SM. Pharmacokinetics of detomidine and its metabolites following intravenous and intramuscular administration in horses. *Eq Vet J* 2009; 41(4): 361-365.
- ³⁵ Exhibit 27: Personal Communication based on RMTC studies, Dr. Scot Waterman, December 13, 2010.
- ³⁶ Exhibit 28: Soma L. Glucocorticoids. *In: Summary of Medication Recommendations*. Provided to the Pennsylvania Horse Racing Commission, Updated September 8, 2010.
- ³⁷ Exhibit 29: Anderson D, Kollias-Baker C, Colahan P. Urinary and serum concentrations of Diclofenac after topical application to horses. *Vet Ther* 2005; 6(1):57-66.
- ³⁸ Exhibit 30: Personal Communication, Dr. Cynthia Cole (*ne* Kollias-Baker).
- ³⁹ Exhibit 31: Personal Communication, Dr. Thomas Tobin. Compromise on regulatory level (HSPA requested 5 ng/ml, MRC 0.8 ng/ml; compromised at 2 ng/ml), April 14, 2011.
- ⁴⁰ Exhibit 32: RMTC Quarterly Report, Fall 2010. RMTC makes withdrawal time recommendations, announces progress of its drug testing initiatives task force. Fall 2010. Available at: www.rmtcnet.net. Accessed October 17, 2011.
- ⁴¹ Exhibit 33: Rumpler MJ, Sams RA, Colahan P. Pharmacokinetics of glycopyrrolate following intravenous administration in the horse. *J Vet Pharmacol Ther* 2011; 34(6): 605-608.
- ⁴² Exhibit 34: Personal Communication based on RMTC studies, Dr. Scot Waterman, November 9, 2010.
- ⁴³ Exhibit 35: Harkins JD, Mundy GD, Stanley S, et al. Absence of detectable pharmacological effects after oral administration of isoxuprine. *Eq Vet J* 1998; 34(4):294-299.
- ⁴⁴ Exhibit 36: Joujou-Sisic K, Andren PE, Bondesson U, et al. A pharmacokinetic study of isoxuprine in the horse. *In: Proceedings of the 11th International Conference of Racing Analysts and Veterinarians, Queensland, Australia. 1996:453-458.*
- ⁴⁵ Exhibit 37: Sams R. Methocarbamol Threshold Study. Powerpoint Presentation. Provided to the RMTC Board of Directors, UC-Davis, October 12, 2010.
- ⁴⁶ Exhibit 38. Association of Racing Commissioners International (ARCI) newsletter, 12/6/2010. Use of zilpaterol earns the most severe penalties. <http://www.arci.com/newsitem.asp?story=1040>. Accessed October 10, 2011.
- ⁴⁷ Exhibit 39: Garcia P, Paris AC, Gil J, et al. Analysis of β -agonists by HPLC/ESI-MSⁿ in horse doping control. *Biomed Chromatogr* 2011; 25:147-154. Wagner SA, Mostrom MS, Hammer CJ, et al. Adverse effects of zilpaterol administration in horses: three cases. *J Eq Vet Sci* 2008; 28(4): 238-243.
- ⁴⁸ Exhibit 40: Wagner SA, Mostrom MS, Hammer CJ, et al. Adverse effects of zilpaterol administration in horses: three cases. *J Eq Vet Sci* 2008; 28(4): 238-243.
- ⁴⁹ Exhibit 41: Sterle J. The facts about Paylean[™] ractopamine for swine. Texas Cooperative Extension Service. Available at: <http://animalscience.tamu.edu/images/pdf/swine/ASWeb-093-TheFactsaboutpayleanractopamineforswine.pdf>. Accessed: October 10, 2011.

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- ⁵⁰ Exhibit 42: Ractopamine and Monensin Plus. Medicated feed label, Blue Bird Feed Mill, January 07, 2008. Available at: www.fda.gov/downloads/AnimalVeterinary/.../UCM202150.pdf. Accessed: October 10, 2011.
- ⁵¹ Exhibit 43: Zilmax plus Rumensin. Food and Drug Administration. Freedom of Information Summary (NADA 141-278). February 15, 2008. Available at: www.fda.gov/downloads/AnimalVeterinary/.../ucm062335.pdf. Accessed October 10, 2011.
- ⁵² Non-published Information. Association of Racing Regulatory Veterinarians private list serve.
- ⁵³ Exhibit 44: Lehner AF, Hughes CG, Harkins JD et al. Detection and confirmation of ractopamine and its metabolites in horse urine after Paylean administration. J Anal Toxicol 2004; 28(4): 226-238.

Minnesota Board of Animal Health
625 Robert St. North
St. Paul, MN 55155 (651) 296-2342

MINNESOTA CERTIFICATE OF VETERINARY INSPECTION

Revised October 2009

41- _____ Unique CVI number, registered to the DVM, goes here

1. Owner/Consignor's Name: _____ Animal owner's name

2. Owner/Consignor's Address: _____ Complete address required

3. Origin Address: (if different than above) _____ Address where animals reside, if different than above

4. Species: Cattle Horse Swine Cat Dog Avian Goat Sheep _____

5. Reason for Movement: Breeding Sale Travel Slaughter Show/Exhibition Feeding

6. Consignee's Name: _____ Animal buyer / new owner's name

7. Consignee's Address: _____ Complete address required

8. Destination: (if different than above) _____ Address where animals will reside, if different than above

9. Carrier's Name and Address: _____

10. Number in Shipment: _____

11. Permit Number: (if required by state of destination)

12. Herd or Flock Status: _____

Disease: _____

Status: _____

Status/Herd/Flock Number: _____

Date of last herd test: _____

13. Identification requirements for movement are set by the State of destination and the USDA. Some animals require Official ID - call the State of destination to find out what they consider official.

14. Travel is marked for companion animals temporarily leaving the state (<30 days) without an ownership change. Consignor and Consignee name and address must be completed.

15. A red circle denotes information that is required to be completed on all CVIs. States of destination may have further requirements.

	Official Ear tag, Tattoo or Other Permanent ID	Brucellosis Vacco. Tattoo	Tuberculosis	Other Test	Other Vaccine or Treatment
	Age	Sex	Brand	Date Injected	Date Result
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					

Certificate of Issuing Veterinarian: I certify as an accredited veterinarian that the described animals have been inspected by me and that they are not showing signs of infectious, contagious and/or communicable disease (except when noted). The vaccination and test results are indicated on the certificate. To the best of my knowledge, the animals listed on this certificate meet the state of destination and Federal Interstate requirements. No warranty is made or implied.

Accredited Veterinarian Signature: _____ Vet Code # _____

Address: _____ Once signed, this is a legal document! _____

Phone No. _____

LS 00167-09 _____

White - with shipment _____ Blue end Pink - copy to Board of Animal Health _____ Yellow - Issuing veterinarian _____

These dates must be less than 10 days apart: _____

Date Inspected: _____ Date Issued: _____

Printed Name: _____ Date of Owner Signature: _____

Phone: _____

Must be forwarded to BAH within 10 days of date issued!

Lynn Hovda

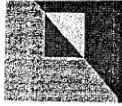
From: Glaser, Linda [Linda.Glaser@state.mn.us]
Sent: Friday, August 20, 2010 1:32 PM
To: Lynn.Hovda@state.mn.us
Cc: Hartmann, Bill; Shaw, Sheryl (USDA); Petrini, Kristine; kaneracingstable@earthlink.net
Subject: Follow up

Dr. Hovda,

We have received the test results on samples collected from horses currently residing in the C2 barn. All samples were negative for equine piroplasmiasis. As we discussed previously, the animals must remain quarantined until tested negative for equine piroplasmiasis on a blood sample collected not less than 30 days after the positive animal was removed from the barn. During this quarantine period, the horses must remain on the Canterbury Park premises. The Board of Animal Health will work with owners or handlers to allow the movement of these horses, if needed, to another Minnesota site where the quarantine can be maintained until the testing requirements are met. It is the decision of the Park management as to what activities the quarantined horses will be allowed to participate in while they remain on the park premises.

Linda C. Glaser, DVM DACVPM

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RACING COMMISSIONERS INTERNATIONAL (RCI)

76 Years of Leadership Advocating Integrity in Racing



Exhibit 3

President's Report: June, 2010

Dear Racing Regulator:

Equine Piroplasmosis (EP) was a major topic of the just concluded American Horse Council meeting in Washington, DC this month. EP is a blood borne parasitic disease that in certain extreme cases can be fatal. EP is not spread by direct contact but requires a movement of blood. It is spread through certain (not all) ticks or the use of contaminated or previously used syringes, drug bottles, dental equipment, even infected lip tattoo equipment.

EP infected horses can develop fever, anemia, yellowing of the membranes in the eyes and mouth, and a dark brown or red tinged urine. Many infected horses never get sick and remain carriers as well as potential sources of infection to other horses.

Horses testing positive for EP are subject to a "lifetime quarantine" in as much as there is no test or treatment yet developed that can clear a horse of this infection. This issue has become pertinent in the southern states, where this issue has come up and the owners of infected horses have sought to enter their horses in events—in our case races.

The good news is that Canada and the United States have been virtually free from instances of EP, otherwise every horse crossing the border would need to be tested in the same ways horses coming from other countries (except Iceland) are required to be tested. The last outbreak of this disease was supposedly eliminated in the United States in 1968 following an extensive control and eradication program conducted by the State of Florida and the United States Department of Agriculture's Veterinary Services bureau (VS).

The bad news is that instances of EP are again showing up in the US and could again in Canada. In 2008 there were twenty positive cases in Florida found in horses associated with unsanctioned racing—i.e. "bush tracks". The next year there were nine cases found in Missouri, again associated with illegal "bush tracks". The spread of EP is believed in these instances to be the result of the use of blood based doping agents in the belief that this will improve performance.

In October last year, 402 positive horses were identified in Texas on a working cattle ranch where heavy tick activity was found. A previously unknown carrier tick, the Cayenne Tick, was found to be the culprit. The USDA says this problem has been found only in South Texas, but the concern is that it will spread.

EP has been identified in at least two equine population types in the US: horses imported prior to 2005 and in a subpopulation of the quarter horse racing industry. The USDA claims that all known infected horses have either been euthanized, died, donated for research, or are being maintained under a lifetime official quarantine.

During the meeting it became apparent that there is a need to educate veterinarians and horsemen to this infection. Often symptoms are misdiagnosed and this is of obvious concern to those trying to maintain the status of the US as an EP free nation. EP is endemic in Mexico, and horses imported absolutely must be tested. The problem here is that horses, just like some people, are not coming into the country legally and the risk is real.

The USDA has conducted testing of horses that originated on the south Texas cattle ranch and had moved to other parts of the US. The testing shows that

these infected horses, now living elsewhere, did not transmit the EP pathogen to other horses, despite living together for multiple years.

Some EP positive horses have been identified through US interstate movement requirements or racing entry requirements at both sanctioned Quarter Horse tracks as well as non-sanctioned facilities. The USDA reports that while only a small number of these horses were found to have the clinical disease, the majority appear normal. They believe these horses were not infected as a result of ticks, but by the reuse of needles, syringes, and other equipment, as well as the administration of blood-origin products supposedly to enhance performance.

Some jurisdictions, like Oklahoma, now require a horse to be tested for EP.

The USDA's Animal Health and Plant Inspection Service has proposed an informational sheet on how to protect one's horse from Equine Piroplasmiasis. This sheet is available on the RCI website as well as the internal SharePoint site. It is important to get this in the hands of as many horsemen and veterinarians as possible.

A common theme articulated to the USDA representatives at the Horse Council meeting was the need to commit research dollars specifically to develop a cure or clearance test for EP positive horses as well as other equine diseases. It was noted that research appropriations specifically for equines have been lacking.

Injury Database:

At least one major jurisdiction has expressed concern to me about the need to access track specific data from the Equine Injury Database being maintained for Thoroughbred racing by The Jockey Club's subsidiary InCompass. The point raised deals with the fact that state employees - i.e. the commission veterinarian—input the data into the database. It is a legitimate argument that this data, if maintained and entered by a state employee, should be accessible to those employing that employee.

Getting the tracks to participate in this program apparently required an agreement that InCompass would not disseminate track specific data, so, requests by commissions directly to the database cannot be directly honored.

Since the tracks are licensed entities and can obtain this information upon request, commissions have the ability to insist that a track request it and make it available to the commission. The other option would be to require that your employees provide you with any information they provide a third party.

The Equine Injury Database is a great project long overdue and an admirable initiative of The Jockey Club and those participating tracks and commissions.

National Racing Compact Project:

The Executive Committee of the United State Trotting Association (USTA) addressed this matter at their most recent meeting. There is formidable opposition from at least two influential directors: Joe Faraldo, President of the Standardbred Owners of New York and Alan Leavitt, a member of the Kentucky Horse Racing Commission.

It looks like the USTA will not be taking a position in support of the compact initiative at this time, even though they have been at the table in preparing the model legislation. If there is a silver lining, it appears that they will not take a position in outright opposition. Certainly Mr. Faraldo is opposing the pending New York legislation in his home state. This is problematic to say the least.

The compact proposal is nothing more than government reorganizing how government does business. The states do not gain any powers they do not now have, nor do they lose any. The unfounded fears that somehow horsemen will lose the ability to lobby for or against something is a red herring that has convinced some to resist this needed reform. Rick Goodell, myself, and others have spent countless hours attempting to address concerns.

Understanding Piroplasmosis

EQUINE PIROPLASMOSIS IS CAUSED BY A protozoan parasite, either *Babesia equi* (formerly known as *Theileria equi*) or *Babesia caballi*. Due to its prevalence in recent U.S. outbreaks, *B. equi* is the focus of this article.

The recent insidious emergence of piroplasmosis, a tick-borne disease, is a striking reminder of the vigilance required to remain free of the class of infectious diseases that include persistence in their pathogenesis. Pathogen persistence is the ability of an infectious organism to remain in the host long-term, even for life, in the absence of easily detectable clinical disease. A critical outcome of persistence is infected populations that are clinically silent reservoirs for transmission.

Infection of equine erythrocytes by *B. equi* leads to variable levels of anemia, fever, anorexia, malaise, and icterus. Although death is possible following infection of a naive horse, it has not been reported in the current U.S. infections. The reasons for clinically silent transmission of *B. equi* are not well understood. Possibilities include missed diagnosis due to the non-specificity of clinical signs; the contributions of parasite virulence and the horse's ability to control parasite replication; the roles of infection prevalence within horses and transmission of competent tick populations; and the overall health status of newly infected horses. These parameters likely contribute collectively to transmission dose and clinical outcome of initial infection.

Although the host-parasite-vector parameters responsible for the level of clinical disease are not well understood, factors likely responsible for re-emergence of *B. equi* infections in the United States are better defined. The historical widespread use of the complement fixation test (CFT) for serological screening of horses moving internationally likely allowed for entrance of infected horses into countries considered free of infection and/or disease. Equine immunoglobulin IgG(T) does not fix complement via the classical pathway and therefore contributes to false-negative CFT results. Movement of infected horses into areas with transmission-competent ticks may lead to additional infections. Transition to the use of

cELISA and polymerase chain reaction (PCR) testing has enhanced detection of clinically silent, persistently infected horses. An important component of infection and disease control in a low-prevalence country such as the United States is treatment of persistently infected horses with the intent of eliminating *B. equi* and removing transmission risk. While a number of drugs have been tested, the majority of data have been derived using imidocarb dipropionate (ID). Published data clearly show that ID is an effective anti-babesial chemotherapeutic that reduces *B. equi* parasitemias associated with acute and persistent infections.

However, controversy exists concerning the ability of ID to completely eliminate *B. equi* from persistently infected horses. Several reasons exist for this controversy. Past use of the CFT to measure the expected decrease in anti-*B. equi* antibody following parasite removal may have given false-negative results, and since several different recommended ID doses and treatment protocols have been used, some may have led to ID-resistant strains. Alternatively, there may be naturally occurring strains or sub-populations of *B. equi* that are resistant to elimination by ID.

Further complicating assessment of chemotherapeutic efficacy in the complete elimination of *B. equi* persistence is the potential of persistence-specific antibody titers, even in the absence of stimulating antigen. Antigen-independent models have been proposed to explain the persistence of long-term antibody titers. These models include memory B lymphocytes with special "memory" qualities that need fewer signals to mature to plasma cells and/or the presence of long-lived, antibody-producing plasma cells. A possible outcome of persistent antibody titers is finding treated horses that are PCR-negative but antibody-positive for *B. equi*, suggesting parasite elimination but antibody persistence. Should such data be forthcoming, consideration must be given to changing the premise that specific antibody titers always indicate *B. equi* infection and transmission risk.

Management options available to owners of *B. equi* and/or *B. caballi* infected horses are evolving and variable depending on location.



Knowing that ticks are required for natural transmission allows for tick-free quarantine as one option. The implementation of this option is related to the practicality of maintaining a tick-free environment and knowledge of the transmission capacity of local ticks. Next, historical and recent data indicate that ID treatment will remove transmission risk from some infected horses; however, a clear understanding of the meaning of the presence of specific antibody in a treated, PCR-negative horse must be defined.

There are at least two explanations for an ID-treated, PCR-negative, antibody-positive horse. First is that a specific antibody may be the result of the length of the infection and long-lived memory B lymphocytes and plasma cells. Second is that it has been hypothesized

that in PCR-negative, antibody-positive horses, parasites are sequestered in, for instance, capillary beds or bone marrow. With time the parasites will emerge in the peripheral blood, and such a horse will re-convert to PCR-positive and remain a transmission risk. It is the opinion of this author that the collective data indicating ID treatment for complete removal of *B. equi* and or *B. caballi* from infected horses are worthy of consideration in non-endemic countries. The final word on ID treatment must await the defining of the transmission risk of treated horses that are PCR-negative but possess persisting specific antibody.

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KENTUCKY

Foal Pneumonia

FOALS, LIKE OTHER YOUNG ANIMALS, ARE especially susceptible to diseases. A review of necropsy cases over a one-year period at the University of Kentucky Livestock Disease Diagnostic Center was conducted to determine common pathologic diagnoses in the foals submitted for necropsy. For this study, all foals 1 day to 6 months of age were included. A total of 272 foals within this age range were submitted and examined in the one-year period. A variety of different pathologic diagnoses were made; however, by far the most common diagnosis was pneumonia.

There were 84 foals diagnosed with pneumonia, which represented 31% of all submitted foals. The cases were arbitrarily separated into age groups of 1 to 6 days, 1 to 4 weeks, and more than 1 to 6 months of age. Forty-two cases (51%) were less than 6 days of age, seven cases (9%) were 1 week to 4 weeks of age, and 33 cases (40%) were 1 to 6 months of age. In two cases the age was not indicated. These data show the first week of life to be a critical time

for the development of pneumonia; however, pneumonia is also problematic in older foals. Fillies comprised 58% of the cases; colts, 42%. There were 71 Thoroughbreds (87%), four Standardbreds, two Quarter Horses, two Miniature Horses, one American Saddlebred, one Hanoverian, and one mixed-breed foal. The high percentage of Thoroughbreds is consistent with the horse population of the area. Thirty-eight of the 84 cases (45%) had other pathologic diagnoses in addition to pneumonia. Common additional conditions included enteritis, septicemia, and fractured ribs.

Pneumonia in this group of foals was commonly associated with bacterial infection. Other causes of foal pneumonia, such as viruses and parasites, were not diagnosed. Of the 84 pneumonia cases, bacteria were isolated from 40 cases, and 44 cases had either no growth (38 cases) or non-pathogenic bacteria (six cases). The foals often had been treated, and prior antibiotic therapy likely contributed to the inability to isolate bacteria even though there



Protect Your Horses from Equine Piroplasmosis

Versión en Español

Equine Piroplasmosis (EP) is a blood-borne parasitic disease that affects horses, ponies, donkeys, mules, and zebras. EP-infected animals can develop fever, anemia, yellowing of the membranes in the eyes and mouth, and dark brown to red-tinged urine. Some animals die from the disease, while others never get sick. Horses with persistent EP infections are carriers of the parasites that cause the disease and are potential sources of infection to other horses.

Since 2008, EP-infected horses have been found in several States. Horses that tested positive for the disease have been quarantined or euthanized, and horses that had contact with infected horses have been tested. The USDA's Animal and Plant Health Inspection Service (APHIS) has developed guidelines for managing infected and exposed horses and is working with EP researchers, equine industry partners, and State animal health authorities to develop additional control strategies. For information on the control of EP in your State, contact your State Animal Health Official or the Federal Area Veterinarian in Charge for your State.

How EP is spread

EP is spread by certain ticks, which move the parasites from one horse to another. Recently, EP has been spread via ticks on a small number of premises in close proximity. An on-going investigation is underway to determine the types of ticks involved. At this point, it does not appear that EP infections via ticks have occurred outside the affected premises. Investigations are ongoing to determine how EP was introduced to U.S. horses.

People can also spread the EP disease agents by reusing needles or syringes between infected and uninfected horses. Dental, tattoo, and surgical equipment can also spread disease if they are not thoroughly cleaned and disinfected between horses. In addition, taking blood from an infected horse—even one that appears healthy—and giving it to an uninfected horse as a transfusion would likely move the disease agent between horses.

Recently, disease spread has occurred through the reuse of needles and/or syringes. There have also been reports during disease investigations of nonveterinarians administering blood transfusions on horses to enhance performance.

What you can do to protect your horses from EP

ALWAYS

- Use a new sterile needle and syringe for all injections, whether into a vein or muscle
- Clean and disinfect equine dental, tattoo, and surgical equipment between horses
- Have any horse that will serve as a blood donor tested for EP
- Contact your veterinarian if your horse is sick and has signs of fever, reduced feed intake, or lethargy
- Check with your State Animal Health Official if you need more specifics about EP

NEVER

- Reuse needles or syringes between horses, even if the horses live on the same facility and appear normal
- Insert a previously used needle into a drug or vaccine multidose bottle
- Use dental, tattoo or surgical equipment that has not been thoroughly cleaned and sanitized
- Use blood from a horse of unknown infectious disease agent status for transfusion

More information on EP is available at:
http://www.aphis.usda.gov/publications/animal_health/content/printable_version/fs_equinep_08.pdf

To find the Federal Area Veterinarian in Charge in your State:
http://www.aphis.usda.gov/animal_health/area_offices/ (click your State on the interactive map).

For a listing of State Animal Health Officials:
<http://www.usaha.org/StateAnimalHealthOfficials.pdf>

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APHIS**Stakeholders Announcement**

Veterinary Services

November 1, 2010

USDA Releases Equine Piroplasmosis Literature Review

Concerns about equine piroplasmosis (EP), a blood-borne disease considered to be foreign to the United States, have prompted educational efforts to inform veterinarians and horse owners about the disease. To further these educational efforts, the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) is releasing *A Literature Review of Equine Piroplasmosis*. This comprehensive report on EP will serve as a resource for veterinarians and others within the equine industry. The report is available at www.aphis.usda.gov/animal_health/animal_diseases/piroplasmosis/downloads/ep_literature_review_september_2010.pdf. No hard copies of the report will be published by USDA-APHIS.

Although EP is primarily transmitted to horses by ticks, this blood-borne disease has been spread mechanically from animal to animal by contaminated blood and intravenous equipment (syringes/needles). Once infected, a horse might not show signs of disease for 7 to 22 days, and some horses may never show any clinical signs. Cases of EP can be mild or acute. In its milder form, EP causes the horse to appear weak and to show a lack of appetite. Acutely affected equids can have fever, anemia, jaundiced mucous membranes, swollen abdomens and labored breathing. EP can also cause a roughened hair coat, constipation and colic.

Other USDA information about EP, including the factsheets "Equine Piroplasmosis" and "Protect Your Horses from Piroplasmosis," is available at www.aphis.usda.gov/animal_health/animal_diseases/piroplasmosis/. Reports of recent EP cases are available from the World Organization for Animal Health (OIE) at www.oie.int/wahis/public.php?page=weekly_report_index&admin=0.

Note to Stakeholders: Stakeholder announcements and other APHIS information are available on the Internet. Go to the APHIS home page at www.aphis.usda.gov and click on the "Newsroom" button. For additional information on this topic, contact Lyndsay Cole at (970) 494-7410, or e-mail lyndsay.m.cole@aphis.usda.gov

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Exhibit 7

A Literature Review of Equine Piroplasmosis

September 30, 2010

www.equine.com

Acknowledgements

This in-depth review of the literature related to equine piroplasmiasis was prepared by Drs. Josie Traub-Dargatz, Barbara Bischoff, Angela James, and Jerome Freier. Sections of this literature review are based on a document previously prepared by the Regionalization Evaluation Services International (RESI) group in the National Center for Import and Export of USDA-APHIS-VS. The National Equine Piroplasmiasis Working Group members and some members of the RESI group reviewed drafts of this document and provided input relating to the content and editorial suggestions.

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A Literature Review of Equine Piroplasmosis

Introduction

Equine piroplasmosis (EP), also referred to as babesiosis, is a disease of equids including horses, donkeys, mules, and zebras. EP is caused by the blood-borne protozoan parasites *Babesia caballi* or *Babesia equi*. These parasites are naturally transmitted from host to host via tick vectors, and dual infection with both organisms has been reported in equids.

In 1998, Mehlorn and Schein proposed a reclassification of *B. equi* to *Theileria equi*. A second group of researchers have proposed that *B. equi* be classified as *Babesia equi* (*incertae sedis*) because based on genomic analysis it belongs in a lineage between *Babesia* and *Theileria* (D. Knowles, pers. comm., 2010). For the remainder of this report the nomenclature of *B. equi* (*incertae sedis*) will be shortened to *B. equi*.¹

EP is endemic in equids in many tropical and subtropical regions, including Africa, the Middle East, Asia, Central and South America, the Caribbean (including Puerto Rico), and Europe. The United States, Canada, Australia, Japan, England, Iceland, and Ireland are not considered endemic areas.

The United States was considered free of EP in 1988 as a result of a joint USDA-Animal and Plant Health Inspection Services (APHIS) and State of Florida eradication program for *B. caballi* that began in 1962 (USDA, 2008).

EP is reportable to the World Organization for Animal Health (OIE) [OIE, 2009a]. The OIE publishes EP status information submitted by member countries, but does not specifically recognize countries as free of EP (OIE, 2009b). Movement requirements for equids usually depend on the perceived risk of disease introduction from importation from a given country by the receiving country. Importing countries may impose their own requirements for pre-import isolation and/or testing of equids from other countries, regardless of the exporting country's self-reported disease status. Similarly, each State in the United States has the authority to impose requirements for equids entering its State. State import regulations are available through the USDA (2010) or by contacting the State Veterinarian for the State of destination (USAHA, 2010).

APHIS regulations pertaining to the importation of animals and animal products into the United States are set forth in 9 CFR Parts 92-99 (USDA, 2009a). Horses are defined in these regulations as horses, asses, mules, and zebras (9 CFR 93.300). Horses for importation are required to undergo quarantine at an APHIS-approved quarantine facility in the United States, or, in the case of Mexican horses, a Mexican facility (9 CFR 93.308 and 93.324). The minimum quarantine duration varies depending on the animal health status of the region of origin of the horse. Conditions for release from quarantine include the absence of evidence of communicable disease, as well as a negative official test for EP. The official test for EP (*B. caballi* or *B. equi*) for importing equids into the United States is the cELISA, as specified by VS Memorandum 291.58 (USDA, 2005). Horses entering the United States from Canada and Iceland are not required to be tested for EP.

In addition, horses for importation must be accompanied by a certificate of a salaried veterinary officer of the national government of the country of origin. The certificate must verify that no cases of EP occurred on the premises of origin or adjoining premises in the 60 days preceding export (9 CFR 93.314). In addition, except for horses from Canada, horses for importation must be certified as having been inspected and found to be free from ectoparasites (USDA, 2009b, 2009c, 2009d). In the United States, EP clinical cases have recently been detected in Florida (August 2008), Missouri/Kansas (June 2009), and Texas (October 2009). Epidemiological investigations of the Florida and Missouri/Kansas outbreaks

¹ Within the historical section the terminology used for EP pathogens and ticks was retained as referred to in the original cited reference.

suggest transmission was by iatrogenic means via needle sharing or blood exchange, not natural transmission via tick vectors.

As of November 2009, New Mexico has required that all horses entering New Mexico racetracks test negative for *B. equi*, and, as of March 8, 2010, a joint surveillance program conducted by the New Mexico Racing Commission and the New Mexico State Veterinarian has identified 16 infected horses. The Texas outbreak appears to be unrelated to those in Florida, Missouri/Kansas, and New Mexico. The source of the Texas outbreak was undetermined as of March 8, 2010, but transmission appears to have occurred on the index ranch over a period of at least 20 years via *Amblyomma cajennense* ticks and possibly *Dermacentor variabilis*. Updates for the investigations are available from OIE (2010).

As of February 2009, the Florida 2008 outbreak was resolved via a thorough investigation, including traceouts, testing, and removal of all infected horses from Florida premises. For the Missouri/Kansas outbreak, five horses infected with EP were euthanized; one infected horse was removed from a premises in Kansas prior to quarantine being initiated; and two infected horses were illegally removed from quarantine in Missouri. The 16 horses identified as infected through New Mexico surveillance have been euthanized or quarantined. The Texas investigation is ongoing, and the index ranch and any premises with horses traced to the index ranch have been quarantined.

The costs of responding to an EP outbreak vary and depend on the extent of response necessary, as well as movement and testing requirements. The joint APHIS–VS and Florida *B. caballi* eradication program initiated in 1962 took 25 years to be considered successful. This effort incurred direct costs of \$12 million for tick inspections, testing, treatment of infected horses, and movement controls (USDA, 2008). When Florida experienced a less extensive EP outbreak due to *B. equi* in 2008, State personnel spent over 4,000 hours making 420 contacts during the investigation. This cost the State an estimated \$150,000 (Short, pers. comm., 2009). The impact of the 2008 EP outbreak on the Florida horse industry is more difficult to estimate, but it may have cost the State's Thoroughbred breeding industry millions of dollars in lost sales and future earnings due to a Canadian ban on importation of horses from Florida during 2008 (Steffanus, 2008). Costs of subsequent outbreaks in other States have yet to be determined.

References—Introduction

- Mehlhorn H, Schein E, 1998. Redescription of *Babesia equi* (Laveran, 1901) as *Theileria equi*. *Parasitol Res* 84(6):467–475.
- OIE, 2009a. Diseases notifiable to the OIE. Available at: http://www.oie.int/eng/maladies/en_classification.htm
- OIE, 2009b. World Animal Health Information Database (WAHID). World animal health information, 2005 and later. Accessed March 10, 2009.
- OIE, 2010. Equine piroplasmiasis, United States of America. Available at: http://www.oie.int/wahis/public.php?page=single_report&pop=1&reported=8530
- Steffanus D, 2008. Unsettling find of piroplasmiasis. *Horse Cap Dig*, Florida Equine Publications, Ocala, FL.
- USAHA, 2010. State Animal Health Officials, 2010. Available at: <http://www.usaha.org/StateAnimalHealthOfficials.pdf>
- USDA–APHIS, 2005. Testing of equidae for import. Veterinary Services Memorandum 291.58.
- USDA–APHIS, 2008. Equine piroplasmiasis. APHIS Factsheet.
- USDA–APHIS, 2009a. Code of Federal Regulations; Title 9, Animals and animal products, ch. 1, 1-199. College Park, MD.
- USDA–APHIS, 2009b. 3-day quarantine: importing a horse into the United States. USDA guide sheet for horses that require a 3-day quarantine. Accessed December 17, 2009. http://www.aphis.usda.gov/import_export/animals/animal_import/equine/equine_import.shtml
- USDA–APHIS, 2009c. 7-day quarantine: importing a horse into the United States. USDA guide sheet for horses that require a 7-day quarantine. Accessed December 17, 2009. http://www.aphis.usda.gov/import_export/animals/animal_import/equine/equine_import7day.shtml

USDA-APHIS, 2009d. 60-day quarantine: importing a horse into the United States guide sheet for horses from countries affected by African horse sickness (AHS). Accessed December 17, 2009.
http://www.aphis.usda.gov/import_export/animals/animal_import/equine/equine_import60day.shtml

USDA-APHIS, 2010. U.S. State and Territory Animal Import Regulations. Available at:
http://www.aphis.usda.gov/import_export/animals/animal_import/animal_imports.states.shtml

Terms Used in This Report

Abiotic—Not associated with or derived from living things. Describes the physical and chemical aspects of an organism's environment such as light, temperature, moisture, and atmospheric gases.

Active stage for tick—Stage in which ticks are seeking hosts, feeding, and mating.

Biotic—Relating to, produced by, or caused by living organisms.

Dormant stage for tick—Inactive nymphal stage, from fall through following spring.

EC50—The concentration of half maximal effective concentration. Refers to concentration of a drug which induces a response halfway between the baseline and maximum after some specified exposure time. Commonly used as a measure of a drug's potency.

Generalist—Generalist species thrive in a wide variety of environmental conditions, making use of many different resources.

Generation time—Time taken for a tick to complete its life cycle from egg to adult.

Immature tick—Larval and nymphal stages of ticks. Ticks are unable to reproduce during these stages.

LD50—An index of toxicity (lethal dose 50 percent). The amount of the substance that kills 50 percent of the test population of experimental animals when administered as a single dose.

Premunity—(a) Resistance to a disease due to the existence of its causative agent in a state of physiological equilibrium in the host; (b) immunity to a particular infection due to previous presence of the causative agent.

Questing—The act of a tick searching for a host. For example, when a tick climbs to the top of vegetation and slowly waves its forelegs while waiting for a passing host.

Current Situation—Prevalence and Geographic Distribution of EP

Review articles and studies can reveal general trends in EP prevalence and distribution, but regional differences must be cautiously interpreted with consideration of study design, sample size, and the tests used to determine infection status. No recent comprehensive data on the worldwide prevalence and geographic distribution of *B. caballi* and *B. equi* are available.

In general, the geographic distributions of *B. caballi* and *B. equi* are similar and include most of the world's tropical and subtropical regions (Brüning, 1996; Friedhoff et al., 1990). The distribution of *B. caballi* extends more often to northern latitudes than *B. equi* (Friedhoff et al., 1990; Friedhoff and Soulé, 1996). Both species of *Babesia* are endemic to many parts of Africa, Europe, the Middle East, and Asia (Brüning, 1996). In most regions of the world where EP is endemic, *B. equi* infections are more prevalent than *B. caballi* infections (Schein, 1988; Friedhoff et al., 1990; de Waal, 1992; Brüning, 1996; Friedhoff and Soulé, 1996; Heuchert et al., 1999; Kerber et al., 1999; Rothschild and Knowles, 2007).

Prior to the 2008 Florida outbreak (OIE, 2009b, 2009c, 2009d), the most recent case of EP on the United States mainland also occurred in Florida in 1978 (OIE, 2009b; USDA, 2009). Other equids infected with EP agents have been detected sporadically in the United States. Typically these cases have occurred in Florida in clinically normal horses with a history of importation into the United States. EP is present in Puerto Rico and the U.S. Virgin Islands (USDA, 2009). Canada is widely considered to be free of EP, although it has been detected sporadically, most recently in 1987 (AVMA, 2006; Center for Food Security and Public Health, 2008; CVO Australia, 2008; USDA, 2008; OIE, 2009a, 2009e). As of March 2009, the EP status of Mexico was listed by the OIE as confirmed infection but no clinical disease (OIE, 2009a).

Few data are available regarding the distribution of *B. caballi* and *B. equi* in Caribbean nations. In one recent study, 83 percent of 93 Thoroughbreds in Trinidad tested seropositive for *B. caballi*, *B. equi*, or both (Asgarali et al., 2007). The seroprevalence of *B. caballi* in this study was substantially higher than that of *B. equi* (69 and 33 percent, respectively).

EP is endemic to most of Central and South America, except in southern regions of Chile and Argentina (Friedhoff and Soulé, 1996). In several studies, large proportions of horses in one or more regions of Colombia, Brazil, and Chile tested positive for *B. caballi*, *B. equi*, or both, as determined by serological or nucleic acid detection methods. In several South American surveys, seroprevalence was higher for *B. equi* than for *B. caballi* (Schein, 1988; Tenter et al., 1988; Pfeifer Barbosa et al., 1995; Heuchert et al., 1999; Kerber et al., 1999; Xuan et al., 2001; Battsetseg et al., 2002; Heim et al., 2007).

EP is endemic to most regions of Africa (Schein, 1988; Friedhoff and Soulé, 1996). The majority of clinical EP cases in Africa are due to *B. equi* infection (Motloang et al., 2008). In one serological study, 80 percent of 6,350 serum samples from various regions of South Africa were positive for *B. equi*, and approximately 50 percent were positive for *B. caballi* (Zweygarth et al., 2002b). Both *B. caballi* and *B. equi* were detected in zebras from two national parks in South Africa by serological and culture methods (Zweygarth et al., 2002a).

B. caballi and *B. equi* are endemic to southern Europe, including Portugal, Spain, France, Belgium, Italy, and the Balkan Peninsula (Schein, 1988; de Waal, 1992; Rothschild and Knowles, 2007). The distribution of *B. caballi* and *B. equi* extends east to Hungary, Romania, and to the southern and western regions of the Commonwealth of Independent States (Schein, 1988; Friedhoff and Soulé, 1996). Most infections that have occurred in regions of Europe where *B. caballi* and *B. equi* are not endemic have been traced back to Spain, France, Italy, or the Commonwealth of Independent States (Rothschild and Knowles, 2007). Across the endemic regions of Europe, *B. caballi* and *B. equi* seroprevalence in equids is highly variable, ranging from about 8 to 35 percent for *B. caballi* and from 20 to 40 percent for *B. equi* (Camacho et al., 2005; Hornok et al., 2007; Acici et al., 2008; Sevinc et al., 2008).

Equine infections with *B. caballi* and *B. equi* are also prevalent in the Middle East and Asia (Donnelly et al., 1980; Schein, 1988; Friedhoff and Soulé, 1996; Rothschild and Knowles, 2007), and both *B. caballi* and *B. equi* are widespread in China and Mongolia (Friedhoff and Soulé, 1996; Xuan et al., 2002; Xu et al., 2003; Boldbaatar et al., 2005; Rothschild and Knowles, 2007). Japan has never reported an occurrence of EP to the OIE and is widely considered to be free of the disease (Friedhoff and Soulé, 1996; Ikadai et al., 2002; Rothschild and Knowles, 2007; OIE, 2009b). However, a recent seroepidemiologic study of more than 2,000 banked horse-serum samples collected for EIA surveillance from 1971 to 1973, found that 5 percent of the samples tested positive for *B. caballi*, and 2 percent were positive for *B. equi*, as determined by ELISA. This finding suggests that *B. caballi* and *B. equi* infections have occurred in Japan (Ikadai et al., 2002).

Neither *B. caballi* nor *B. equi* has become established in Australia or New Zealand (de Waal, 1992; Brüning, 1996; Friedhoff and Soulé, 1996; Martin, 1999; AVMA, 2006; Rothschild and Knowles, 2007; Center for Food Security and Public Health, 2008; CVO Australia, 2008; USDA, 2008a). Sporadic occurrences in Australia in the 1950s, 1960s, and 1970s were attributed to introduction by imported horses (CVO Australia, 2008). According to OIE data, EP occurred in Australia most recently in 1976. New Zealand has never reported the occurrence of EP to the OIE (OIE, 2009e, 2009f).

References—Prevalence and Geographic Distribution of EP

- Acici M, et al., 2008. Seroprevalence of equine babesiosis in the Black Sea region of Turkey. *Parasitol Int* 57(2):198–200.
- American Veterinary Medical Association, 2006. Equine piroplasmiasis background, August 25, 2006. Available at: http://www.avma.org/reference/backgrounders/equine_piroplasmiasis_bgnd.asp
- Asgarali Z, et al., 2007. A serological study of *Babesia caballi* and *Theileria equi* in Thoroughbreds in Trinidad. *Vet Parasitol* 144(1-2):167–171.
- Battsetseg B, et al., 2002. Detection of natural infection of *Boophilus microplus* with *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. *Vet Parasitol* 84(1-2):75–83.
- Boldbaatar D, et al., 2005. Epidemiological study of equine piroplasmiasis in Mongolia. *Vet Parasitol* 127(1):29–32.
- Center for Food Safety and Public Health, 2008. Equine piroplasmiasis, August 29, 2008. Available at: http://www.cfsph.iastate.edu/Factsheets/pdfs/equine_piroplasmiasis.pdf
- Camacho AT, et al., 2005. *Theileria (Babesia) equi* and *Babesia caballi* infections in horses in Galicia, Spain. *Trop Anim Health Prod* 37(4):293–302.
- Brüning A, 1996. Equine piroplasmiasis: an update on diagnosis, treatment and prevention. *Br Vet J* 152(2):139–151.
- de Waal DT, 1992. Equine piroplasmiasis: a review. *Br Vet J* 148(1):6–14.
- Donnelly J, et al., 1980. A comparison of the complement fixation and immunofluorescent antibody tests in a survey of the prevalence of *Babesia equi* and *Babesia caballi* in horses in the Sultanate of Oman. *Trop Anim Health Prod* 12(1):50–60.
- Friedhoff KT, Soulé C, 1996. An account on equine babesiosis. *Rev Sci Tech Off int Epiz* 15(3):1191–1201.
- Friedhoff KT, Tenter AM, Müller I, 1990. Haemoparasites of equines: impact on international trade of horses. *Rev Sci Tech* 9(4):1187–1194.
- Heim A, et al., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. *Parasitol Res* 102(1):63–68.
- Heuchert CMS, et al., 1999. Seroepidemiologic studies on *Babesia equi* and *Babesia caballi* infections in Brazil. *Vet Parasitol* 85(1):1–11.
- Hornok S, et al., 2007. Serological evidence for *Babesia canis* infection of horses and an endemic focus of *B. caballi* in Hungary. *Acta Vet Hung* 55(4):491–500.
- Ikadai H, et al., 2007. Seroepidemiologic studies on *Babesia caballi* and *Babesia equi* infections in Japan. *J Vet Med Sci* 64(4):325–328.
- Kerber CE, Ferreira F, Pereira MC, 1999. Control of equine piroplasmiasis in Brazil. *Onderstepoort J Vet Res* 66(2):123–127.
- Martin R, 1999. Equine piroplasmiasis: the temporary importation of seropositive horses into Australia. *Aust Vet J* 77(5):308–309.

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- Motloang MY, et al., 2008. Prevalence of *Theileria equi* and *Babesia caballi* infections in horses belonging to resource-poor farmers in the north-eastern Free State Province, South Africa. Onderstepoort. *J Vet Res* 75(2):141–146.
- Office of the Chief Veterinary Officer Australia—Disease Preparedness Section, 2008. Exotic animal diseases bulletin: equine piroplasmiasis. *Aust Vet J* 86(11):N20–N21.
- OIE, 2009a. World Animal Health Information Database (WAHID). World animal health information, 2005 and later. Accessed March 10, 2009.
- OIE, 2009b. Follow-up report no. 5, equine piroplasmiasis, Florida, United States. OIE ref. no. 7820, February 23, 2009.
- OIE, 2009c. Follow-up report no. 4, equine piroplasmiasis, Texas, United States. OIE ref. no. 8682, November 23, 2009.
- OIE, 2009d. Follow-up report no. 4, equine piroplasmiasis, Missouri, United States. OIE ref. no. 8406, September 15, 2009.
- OIE, 2009e. Handistatus II. World Animal Health Information, 2004 and earlier. Accessed March 10, 2009.
- OIE, 2009f. World Animal Health Information Database (WAHID). Disease timelines: Equine piroplasmiasis. Accessed March 10, 2009.
- Pfeifer Barbosa I, et al., 1995. Epidemiological aspects of equine babesiosis in a herd of horses in Brazil. *Vet Parasitol* 58(1-2):1–8.
- Rothschild C, Knowles D, 2007. Equine piroplasmiasis. In *Equine Infectious Diseases*, ed. Sellon DC, Long Mt. Saunders, Elsevier, St. Louis, MO. 465–473.
- Schein E, 1988. Equine babesiosis. In *Babesiosis of Domestic Animals and Man*, ed. Ristic M; CRC Press, Boca Raton, FL. 197–208.
- Sevinc F, et al., 2008. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. *Vet Parasitol* 156(3-4):173–177.
- Tenter AM, et al., 1988. Prevalence of piroplasmiasis in equines in the Colombian province of Cordoba. *Trop Anim Health Prod* 20(2):93–93.
- USDA—APHIS, 2008. Equine piroplasmiasis. APHIS Factsheet:
- USDA—APHIS, 2009. Animal health monitoring and surveillance. Status of reportable diseases in the United States. Available at: http://www.aphis.usda.gov/vs/nahss/disease_status.htm
- Xu Y, et al., 2003. Seroepidemiologic studies on *Babesia equi* and *Babesia caballe* infections in horses in Jilin province of China. *J Vet Med Sci* 65(9):1015–1017.
- Xuan Xet al., 2001. Diagnosis of equine piroplasmiasis in Brazil by serodiagnostic methods with recombinant antigens. *J Vet Med Sci* 63(10):1159–1160.
- Zweygarth E, Lopez-Rebollar LM, Meyer P, 2002a. *In vitro* isolation of equine piroplasms derived from Cape Mountain zebra (*Equus zebra zebra*) in South Africa. Onderstepoort. *J Vet Res* 69(3):197–200.
- Zweygarth E, et al., 2002b. Culture, isolation and propagation of *Babesia caballi* from naturally infected horses. *Parasitol Res* 88(5):460–462.

Historical Perspective

Note: For this section, names of ticks and causative agents used in original reports are maintained.

History of EP organisms' discovery and early literature

Before 1901, EP was not recognized as a distinct disease and was often confused with other diseases. EP has been called anthrax fever, bilious fever or bilious form of horse sickness, biliary fever, and equine malaria (Roberts et al., 1962). EP was referred to as equine malaria because the clinical signs of the hemoparasitic infection observed in equids in Pretoria, South Africa, were similar to malaria infection (plasmodiidae) found in humans (Theiler, 1903). Based upon morphology, the hemoparasite was classified with other pear-shaped protozoa known as piroplasms, and was named *Piroplasma equi* (Laveran, 1901).

In the early 20th century, South African veterinarian Sir Arnold Theiler unsuccessfully attempted to transmit the disease agent from affected horses to unaffected horses via blood transfusion. Theiler speculated that he was unable to create the disease because native horses were already immune to it or, perhaps because the disease agent required a tick vector to be infective (Theiler, 1902). Theiler also reported that the disease did not appear to be directly contagious and that it was recognized most often in horses imported to South Africa from New Zealand, England, Australia, and the Argentine Republic. Although it did not appear that the disease was spread by direct contact, Theiler indicated that epizootics (epidemics) of equine malaria could occur when large numbers of susceptible animals were present. In addition, the disease was recognized more frequently in summer, especially during the rainy season. Theiler also indicated they experience more than one episode of clinical disease. In 1912, Nuttall and Strickland (1912) discovered that EP could be caused by two different agents, *B. equi* and *B. caballi*.

When Giemsa's, Leischemann's, or Wright's stains are used, *B. caballi* is characterized by two pear-shaped, basophilic-staining bodies approximately 4 μ in length in a single red blood cell (Retief, 1964). In a blood smear, *B. caballi* is much larger than *B. equi*. The intraerythrocytic form of *B. equi* may appear as a Maltese cross formation, ameboid, or signet-ring shaped with a clear halo and about 2 μ in length (Retief, 1964). During the Florida *B. caballi* outbreak in the early 1960s, blood samples obtained from jugular veins and from ear notches of 136 horses were compared because some investigators thought that blood obtained from a tissue sample (ear notch), and thus from capillaries, was more likely to contain the parasites. Of the 136 samples tested, 17 had *B. caballi* in red blood cells. In no instance did samples taken from ear notches yield a superior ability to detect the organism than samples taken from jugular veins (Sippel et al., 1962).

Sippel and others (1962) described a technique for concentrating affected erythrocytes, which entailed mixing equal parts of 2-percent sodium citrate with the blood to be tested followed by centrifuging at 500 to 700 rpm for 3 to 5 min. The supernatant fluid was then decanted and recentrifuged at 1,500 to 2,000 rpm for 15 to 20 min. The supernatant fluid was discarded and smears were prepared from the sediment. The infected cells were lighter in weight than normal cells, enabling the method described above to concentrate the infected erythrocytes.

After the initial discovery of the causative agents for EP, it was confirmed that the disease was transmitted by ticks. Although many species of ticks reportedly transmitted the disease agent in South Africa, red ticks (*Rhipicephalus evertsi*) were the main vectors. Most horses in South Africa that were not stabled were carriers of the disease agent but seldom developed signs of disease because they were immune. In fact, only one clinical case of babesiosis was found during a 3-year period, in a horse on the veldt; however, 50 cases were found in stabled Thoroughbreds that had been de-ticked regularly (Retief, 1964). This report suggests that the de-ticked horses were susceptible to repeat infections as they did not develop a lasting immunity to the disease agent(s).

Premunity to EP in horses depends on the presence of the parasite in the body and lasts 6 months to 1 year. If, however, an immune horse is bitten by another infected tick during this period, a further period of premunity is conferred, which may explain why horses living in the veldt in South Africa become lifelong premune carriers. It has also been reported that foals in endemic areas are generally immune for the first year of life; presumably resistance is acquired through colostral transfer. If a foal is exposed to infected ticks while still immune from passive acquired antibodies, it acquires active immunity (Retief, 1964).

History of EP in the United States

The first reported infections of *B. caballi* and *B. equi* in U.S. equids occurred in 1961 and 1964, respectively. In August 1961, a private veterinary practitioner examined a horse in Dade County, Florida, with clinical signs consistent with equine infectious anemia (EIA) [Strickland and Gerrish, 1964]. Blood from the horse was submitted to a private laboratory, and intraerythrocytic organisms tentatively diagnosed as *B. caballi* were found. This diagnosis was confirmed at the USDA Animal Disease and Parasitological Research Laboratory in Beltsville, Maryland, and at the National Animal Disease Laboratory in Ames, Iowa. The mode and time of introduction of *B. caballi* into the United States was reported as unknown by one author (Knowles et al., 1966) and speculated by another to be the result of the importation in 1959 of 50 Cuban Walking Horses into Davie, Florida in 1959 (Sippel et al., 1962; Stiller and Coan, 1995). These Cuban Walking Horses were then sold to local individuals as privately owned mounts and were placed in various boarding facilities in the Miami-Fort Lauderdale area. Knowles speculated that EP might have gone undiagnosed for years because it was confused with EIA (Knowles et al., 1966). Initially, the only means for diagnosis were clinical signs consistent with the disease and the detection of the parasite in erythrocytes, using specific staining techniques and confirmation through animal inoculation studies (Roberts et al., 1962).

In Florida, from 1961 through September 1963, 141 cases of EP were detected. Infections in horses were diagnosed on 103 Florida premises, based on identification of *B. caballi* via blood smears. The majority of these cases were found in Broward and Dade Counties, which contained large populations of the tropical horse tick *Dermacentor nitens* (Strickland and Gerrish, 1964). These counties also had large equine populations, particularly pleasure horses kept at pasture where tick exposure was likely. Veterinarians in the counties had access to high-quality diagnostic facilities, and the system for reporting cases was very good (Strickland and Gerrish, 1964). However, it is likely that the number of cases was higher than reported because some infected animals were not tested due to lack of clinical signs following resolution of acute parasitemia, and some owners were reluctant to report sick horses due to fear of the regulatory response, which included quarantine. In 1964, a mixed infection of *B. caballi* and *B. equi* was found in the blood of a Florida horse (Ristic et al., 1964), and in 1965 a pure infection of *B. equi* was discovered in an equid in the United States in Florida (Knowles et al., 1966).

By 1969, four *B. equi* infections had been diagnosed in the United States—three in Florida and one in New Jersey. The New Jersey horse had developed signs of EP after importation from Europe. Experiments indicated that the ticks detected on horses in Florida as of 1969 were not transmitting *B. equi* (Taylor et al., 1969). By 1969, EP cases had been identified in five States (Taylor et al., 1969). All *B. caballi* cases outside Florida occurred in horses that had come from Florida, Puerto Rico, or the U.S. Virgin Islands (Taylor et al., 1969; Coffman, 1997). Anemia, fever, and edema were the most commonly reported clinical signs of EP due to *B. caballi* infection. Taylor and others (1969) suggested several reasons for the large number of cases of EP in 1968: the advent of the complement fixation test (CFT) to diagnose the infection; the fact that practicing veterinarians were becoming more aware of the disease and were pursuing a diagnosis; increased tracing by State and Federal animal health officials; and mild weather with greater than average spring rainfall in Florida, which likely contributed to the tick population. It should be noted that the number of cases reported by Taylor differ from the number reported by Coffman. Taylor's source for the number of cases was unpublished data from R.C. Knowles, Chief Staff Veterinarian, Equine Diseases, USDA-ARS, Animal Health Division, Hyattsville, Maryland. Coffman did not indicate the source of the data he reported.

EP cases from 1962 through 1971, as reported by Taylor et al. (1969) and Coffman (1997)

Year	State	Number of Horses with <i>B. caballi</i> (Taylor et al., 1969)	Number of Horses with EP Infection* (Coffman, 1997)
1962	Florida	127	56
	Georgia	4	4
1963	Florida	14	94
1964	Florida	4	4
1965	Florida	4	31
1966	Florida	7	30
1967	Florida	4	66
1968	Florida	175	301
	New Jersey	2	2
	North Carolina	2	2
	Mississippi	1	1
1969	Arkansas	2	1
	Florida	37 (through May)	146
	New Jersey	4 (through May)	4
	Tennessee	26 (through May)	26
1970	Florida	Not reported	160
1971	Florida	Not reported	243

*Report agent not specified in original.

Dermacentor nitens, the tropical horse tick, was the only type of tick identified in *B. caballi* transmission in Florida in the 1960s (Retief, 1964; Knowles et al., 1966). This tick was first described by Neumann in 1897 from collections taken in Jamaica and Santo Domingo (Strickland and Gerrish, 1964). In 1901, the tick was identified in Guatemala, Venezuela, and Puerto Rico. Since then, *D. nitens* has been reported in most Caribbean countries, Central America, Mexico, part of South America, Florida, and several counties in south Texas. The tick was first reported in the United States around 1908 in Cameron County in southern Texas. *D. nitens* has specific temperature and humidity requirements which restricted its geographic distribution. *D. nitens* has been found in the ears of equids as well as the nasal diverticulum, mane, perineum, and along the ventral midline.

In one report, a case fatality rate of about 10 percent was reported among 52 horses in the United States diagnosed with *B. caballi* infection (Sippel et al., 1962). Taylor speculated that some of the fatalities attributed to EP in these reports may have been the result of a concurrent EIA infection. Of the four

horses diagnosed with *B. equi* infection in the United States in the 1960s, two showed signs of disease and none died from the infection (Taylor et al., 1969).

In 1962 a joint equine piroplasmiasis outbreak control task force was formed, which operated until 1978 (Coffman, 1997). In 1962 Florida implemented a regulation that designated EP as a reportable disease and gave the Florida Department of State authority to carry out the objectives of the task force. The Florida EP Control Program involved horse testing, tick surveillance, quarantine of EP-positive horses and the premises on which positive horses or ticks were found, treatment of both clinical cases and inapparent carriers, and treatment of tick-infested horses and premises. EP-positive horses were identified with a lip tattoo or brand that included a *P* and an assigned number (Florida Department of State, 1962). The initial U.S. outbreak was reportedly brought under control in 1971 (Coffman, 1997). From 1962 to 1971, 1,150 EP-positive horses were identified in Florida, and 40 EP-positive horses were found in other States (Coffman, 1997). All EP cases found in States other than Florida were traced to horses originating in Florida, Puerto Rico, or the U.S. Virgin Islands.

The initial U.S. outbreak was reportedly brought under control in 1971 (Coffman, 1997). EP due to *B. caballi* was brought under control by eliminating *D. nitens* via pesticides and by administering babesiacidal drugs to known positive horses identified by CFT. The enzootic area included Florida's Dade, Broward, and Palm Beach Counties. Bryant and others (1969) reported that numbered hoof brands were used to identify EP-positive horses. Brands were renewed every 3 to 4 months.

In an effort to protect the race horse population from EP, the Florida Racing Commission required that: (1) horses arriving in Florida for racing season be inspected for ticks upon entry into the race tracks; (2) race horses be lodged at race tracks or approved stables (where vector control was enforced); and (3) hay and bedding used at the tracks in Florida could not originate from premises in the counties where EP had been diagnosed (Knowles et al., 1966).

Florida continued to operate a tick surveillance and treatment program until 1988 (Coffman, 1997). From 1974 to 1984, only 15 new cases of EP were found in Florida. In 1984, 293 Paso Fino horses at 19 different locations in the State were tested under court order. Of the 293, 35 (12 percent) tested EP positive.² All but one of the positive horses had been legally imported in 1983 or 1984 from Puerto Rico and South America. All of the EP-positive horses were either exported or treated until they tested negative (Coffman, 1997).

The 1994 Florida State Administrative Code required all horses from EP endemic areas to test negative for both *B. caballi* and *B. equi* prior to shipment to Florida and be quarantined and retested between 30 and 60 days after arrival in Florida. After the requirement to retest was implemented, EP-positive² horses were found, usually at low titers on the CFT. These horses were required to remain under quarantine and were treated at the owner's expense or exported (Coffman, 1997). In addition, horses residing on premises where an EP-positive horse had been identified were inspected for ticks by State personnel; if ticks were found, the owner was required to treat all animals and the premises for ticks in accordance with guidelines established by the Department of Agriculture in Florida. The estimated cost for controlling the EP outbreak that began in the 1960s in Florida was \$7 to 8 million, although no details were provided as to how the estimate was made (Coffman, 1997).

History of U.S. import testing related to EP

Import testing of equids for the presence of antibodies to EP agents using the CFT began in October 1970. In 1979, an APHIS proposed rule (Federal Register, 1979) called for amending regulations to eliminate the requirement that horses must have a negative EP test before being imported into the United States. The rule change suggested that the procedure was no longer necessary and its elimination would reduce unnecessary time and expense associated with the importation of horses. The proposed rule also stated that based on a review: "(1) the tick vectors of this disease are severely limited in number and

² The parasite (*B. equi* or *B. caballi*) was not specified in the cited report.

distribution in this country; (2) the potential for disease dissemination without the availability of vectors is markedly lower than with other forms of communicable diseases; (3) many animals with positive test reactions do not show symptoms of the disease; (4) many horses that otherwise meet the import requirements are denied entry because they carry antibodies; (5) the presence of antibodies does not always mean there is infection in animals; and (6) the disease has not been shown to be a significant economic threat to the United States, nor has it been considered a significant economic threat to other countries."

Based on this proposed rule the Infectious Diseases of Horses Committee of the U.S. Animal Health Association stated in their 1979 proceedings, "The Committee wishes to iterate its strong urging that this import requirement be continued." The committee also addressed the USDA procedure for importation and quarantine of horses from Mexico that were consigned to slaughter; the Committee indicated, "it felt that the controls over these imported animals before going to the final destination was not adequate and the Committee opposes any changes in rules relating to the identification and final disposition of these animals."

A notice of withdrawal of the proposed rule from APHIS was posted and effective in September 1980 (Federal Register, 1980). This notice reported that an EP panel held public meetings to solicit public comment, especially from experts that had personal experience with EP. The panel recommended that, based on the evidence received, the EP test requirement should not be eliminated. Ten comments were received during a 60-day comment period. The majority of comments supported maintaining the testing requirement. Once completed, the review by the EP panel indicated that the CFR Part 92.11(d) and 92.34(c) would not be amended at that time. The CFT was used as the official entry test for imported horses until August 2005 when the competitive enzyme-linked assay (cELISA) was implemented as the official test (USDA, 2009).

References—History

- Bryant JE, Anderson JB, Willers KH, 1969. Control of equine piroplasmis in Florida. *J Vet Med Assoc* 154(9):1034–1036.
- Coffman LM, 1997. Equine piroplasmis. United States Animal Health Association Proceedings. 253–259.
- Federal Register, 1979. Proposed Rule 9 CFR Part 92, April 27, 1979. 44(83):24863-24864.
- Federal Register, 1980. 45 FR 64196 Notice of Withdrawal, September 29, 1980. 45.
- Florida Department of State, 1962. Florida Administrative Code, Rule 5C-14.005. Accessed January 1, 2009: <http://www.flrules.org/gateway/ChapterHome.asp?Chapter=5C-14>
- Knowles DP, Mathis RM, Bryant JE, Wilkens KH, 1966. Equine piroplasmis. *J Am Vet Med Assoc* 148:407–410.
- Nuttall GHF, Strickland C, 1912. On the occurrence of two species of parasites in equine piroplasmis or biliary fever. *Parasitol* 5:65–96.
- Retief GP, 1964. A comparison of equine piroplasmis in South Africa and the United States. *J Am Vet Med Assoc* 145:912–916.
- Ristic M, Opperman J, Sibinovic S, Phillips TN, 1964. Equine piroplasmis—a mixed strain of *Piroplasma caballi* and *Piroplasma equi* isolated in Florida and studied by fluorescent-antibody technique. *Am J Vet Res* 25:15–23.
- Roberts ED, Morehouse LG, Gainer JH, McDaniel HA, 1962. Equine piroplasmis. *J Am Vet Med Assoc* 141:1323–1329.
- Sippel WL, Cooperrider DE, Gainer JH, Allen RW, Mouw JEB, Tiegland MB, 1962. Equine piroplasmis in the United States. *J Am Vet Med Assoc* 141:694–698.
- Stiller D, Coan ME, 1995. Recent developments in elucidating tick vector relationships for anaplasmosis and equine piroplasmis. *Vet Parasitol* 57:97–108.
- Strickland RK, Gerrish RR, 1964. Distribution of the tropical horse tick in the United States, with notes on associated cases of equine piroplasmis. *J Am Vet Med Assoc* 144:875–878.
- Taylor WM, Bryant JE, Anderson JB, Wilkens KH, 1969. Equine piroplasmis in the United States—a review. *J Am Vet Med Assoc* 155:915–919.
- Theiler A, 1902. Equine malaria. *J Comp Path and Therap* 15:40–54.

Theiler A, 1903. Equine malaria and its sequelae. *J Comp Path and Therap* 16:97–120.
USDA, 2009. Seroprevalence of equine piroplasmosis disease agents in the United States.
<http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/equine/EP%20info%20sheet.pdf>

Etiology of EP Agents: Life Cycle and Transmission

B. caballi and *B. equi* are protozoan organisms and are obligate intracellular parasites of blood cells (Schein, 1988; Friedhoff and Soulé, 1996). Equids are natural hosts of *B. caballi* and *B. equi*, and ixodid ticks are natural vectors (Uilenberg, 1995; Friedhoff and Soulé, 1996; Rothschild and Knowles, 2007). *B. caballi* and *B. equi* parasites have been detected in dogs; however, the epidemiologic significance of these detections is unknown (Criado-Fornelio et al., 2003; Beck et al., 2009). The lifecycles of *B. caballi* and *B. equi* are similar and include developmental stages in both the equine host and the vector tick. The life cycles of both parasites include sporozoites (an asexual infective stage), merozoites (an asexual blood stage), and gametocytes (a sexual blood stage).

Ticks are biologic vectors and become infected through ingestion of gametocyte-containing erythrocytes (red blood cells) while taking a blood meal from an infected host (Uilenberg, 2006). In the ticks' digestive tracts, the gametocytes develop into gametes, which fuse to form zygotes (Mehlhorn and Schein, 1998; Ueti et al., 2003; Uilenberg, 2006). *B. caballi* zygotes multiply and invade numerous tissues and organs of the ticks, including the ovaries, but not, initially, the salivary glands. *B. caballi* infection can be passed transovarially to the next tick generation, and development to an infective stage for equids occurs in the salivary glands of immature and adult ticks (Uilenberg, 2006; Rothschild and Knowles, 2007). In contrast, *B. equi* zygotes develop into kinetes, which invade the ticks' hemolymph and salivary gland cells (Mehlhorn and Schein, 1998; Ueti et al., 2003; Uilenberg, 2006; Rothschild and Knowles, 2007). Further development to a stage of *B. equi* infective for equids is generally complete within 6 to 24 days after the ticks are infected. The transovarial transmission of *B. caballi* within *Anocentor nitens* is epidemiologically significant in that such ticks are an additional reservoir for transmission, in addition to the persistently infected horse (Rothschild and Knowles, 2007).

An equid can be infected when tick saliva containing infective stages of the parasites is injected into the equid while a tick is feeding (Uilenberg, 2006; Rothschild and Knowles, 2007). *B. caballi* development in the equid host does not include a lymphocyte stage (Friedhoff and Soulé, 1996; Uilenberg, 2006; Rothschild and Knowles, 2007). Although two reports suggested that the initial development of *B. equi* in the equid host occurs in the lymphocytes (white blood cells) [Mehlhorn and Schein, 1998; Uilenberg, 2006] based on in vitro studies another research group has failed to find a lymphocyte phase of *B. equi* infection in vivo in the horse (Knowles, pers. comm., 2010). Importantly, lymphocyte transformation is not a component of *B. equi* infection as is the case in bovine theileriosis, thus suggesting *B. equi* does not meet one of the criteria for reclassification as *Theileria equi* (D. Knowles, pers. comm., 2010). Further development and asexual reproduction occurs in erythrocytes (Friedhoff and Soulé, 1996; Uilenberg, 2006).

Iatrogenic transmission of the parasites can occur via the reuse of needles, syringes, and through blood transfusions from untested, infected donor horses. Sharing equipment, such as dental or tattoo equipment, that may be contaminated with blood is also a potential pathway for transmission.

References—Etiology of EP

- Beck R, et al., 2009. Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. *Int J Parasitol* 39(7):843–848.
- Criado-Fornelio A, et al., 2003. Molecular studies on *Babesia*, *Theileria*, and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. *Vet Parasitol* 113(3-4):189–201.
- Friedhoff KT, Soulé C, 1996. An account on equine babesiosis. *Rev Sci Tech Off Int Epiz* 15(3):1191–1201.
- Mehlhorn H, Schein E, 1998. Redescription of *Babesia equi* (Laveran, 1901) as *Theileria equi*. *Parasitol Res* 84(6):467–475.
- Rothschild C, Knowles D, 2007. Equine piroplasmiasis. In *Equine Infectious Diseases*, ed. Sellon DC, Long Mt. Saunders, Elsevier, St. Louis, MO. 465–473.
- Schein E, 1988. Equine babesiosis. In *Babesiosis of Domestic Animals and Man*, ed. Ristic M. CRC Press, Boca Raton, FL. 197–208.

-
- Ueti MW, et al., 2003. Expression of equi merozoite antigen 2 during development of *Babesia equi* in the midgut and salivary gland of the vector tick *Boophilus microplus*. *J Clin Microbiol* 41(12):5803–5809.
- Uilenberg G, 1995. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet Parasitol* 57(1-3):19–41.
- Uilenberg G, 2006. *Babesia*—a historical overview. *Vet Parasitol* 138(1-2):3–10.

Pathogenesis and Clinical Findings of EP

EP should be included in the differential diagnosis for equids with anemia, jaundice, fever, and signs of chronic inflammatory disease. However, clinical signs are variable and nonspecific, and EP cannot be diagnosed based on clinical signs alone (Rothschild and Knowles, 2007; Center for Food Security and Public Health, 2008). *B. caballi* and *B. equi* infections cannot be distinguished clinically, but differentiation between the two infections may be important for successful treatment and control (Rothschild and Knowles, 2007).

The incubation period, or time from infection to manifestation of clinical signs, ranges from 10 to 30 days for *B. caballi* and 12 to 19 days for *B. equi* (de Waal, 1992). Infection can result in a variety of clinical signs. The course of the disease may be peracute, acute, subacute, or chronic (Rothschild and Knowles, 2007). Peracute and acute signs may include fever, jaundice, anemia, hemoglobinuria, bilirubinuria, digestive or respiratory signs, and occasionally death. Equids with subacute piroplasmiasis may display anorexia, lethargy, weight loss, anemia, limb edema (swelling), poor performance, increased heart and respiratory rates, and splenomegaly. Chronic piroplasmiasis is clinically indistinguishable from other chronic inflammatory diseases and generally presents with nonspecific signs, such as inappetence, poor body condition, and poor performance. Anemia may be minimal or absent in equids with chronic or persistent infection; these animals are termed carriers and are reservoirs for tick and iatrogenic transmission (Rothschild and Knowles, 2007).

The majority of horses seropositive for *B. equi* or *B. caballi* are carriers which, by definition, are persistently infected with EP pathogens but show no clinical signs and serve as a source of infection for other equids throughout their lives (Rothschild and Knowles, 2007). Carrier mares can transmit *B. equi* to their offspring, resulting in abortions, stillbirths, neonatal disease or death, or carrier offspring (de Waal, 1992; Lewis et al., 1999; Phipps and Otter, 2004; Allsopp et al., 2007; Rothschild and Knowles, 2007). Persistent infection of mares by *B. equi* has been reported to be a very common cause of equine abortion in endemic regions; in a study of Thoroughbred mares in South Africa up to 11 percent of abortions were attributed to *B. equi* infections (de Waal, 1992; Lewis et al., 1999). In addition to the negative effects of *B. equi* infections on equine reproduction, persistent infection with EP pathogens can also adversely affect performance. Among equine athletes, seropositive horses may have decreased performance compared with seronegative horses and may be at risk for developing overt clinical disease or even sudden death (Hailat et al., 1997; Rhalem et al., 2001; Rothschild and Knowles, 2007).

Mortality due to EP pathogen infection ranges from 5 to 10 percent among horses native to endemic regions, depending on the pathogen strain, the health of the horse, and treatment (Rothschild and Knowles, 2007). Among naïve mature horses introduced into endemic regions, the case fatality rate commonly exceeds 50 percent (Maurer, 1962; Rothschild and Knowles, 2007). Importation of infected horses into regions where populations of naïve horses and vectors are present may also result in high mortality among indigenous horses. For example, importation of infected horses into southern France resulted in an EP outbreak characterized by a 69-percent case fatality rate among untreated indigenous horses (Maurer, 1962).

References—Pathogenesis and Clinical Findings of EP

- Allsopp MT, Lewis BD, Penzhorn BL, 2007. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. *Vet Parasitol* 148(2):130–136.
- Center for Food Security and Public Health, 2008. Equine piroplasmiasis. Available at: http://www.cfsph.iastate.edu/Factsheets/pdfs/equine_piroplasmiasis.pdf
- de Waal DT, 1992. Equine piroplasmiasis: a review. *Br Vet J* 148(1):6–14.
- Hailat NQ, et al., 1997. Equine babesiosis associated with strenuous exercise: clinical and pathological studies in Jordan. *Vet Parasitol* 69(1-2):1–8.
- Lewis BD, Penzhorn BL, Volkmann DH, 1999. Could treatment of pregnant mares prevent abortions due to equine piroplasmiasis? *J S Afr Vet Assoc* 70(2):90–91.
- Maurer FD, 1962. Equine piroplasmiasis—another emerging disease. *J Am Vet Med Assoc* 141:699–702.

-
- Phipps LP, Otter A, 2004. Transplacental transmission of *Theileria equi* in two foals born and reared in the United Kingdom. *Vet Rec* 154(13):406–408.
- Rhalem A, et al., 2001. Validation of a competitive enzyme-linked immunosorbent assay for diagnosing *Babesia equi* infections of Moroccan origin and its use in determining the seroprevalence of *B. equi* in Morocco. *J Vet Diagn Invest* 13(3):249–251.
- Rothschild C, Knowles D, 2007. Equine piroplasmiasis. In *Equine Infectious Diseases*, ed. Sellon DC, Long Mt. Saunders, Elsevier, St. Louis, MO. 465–473.

Equine Immune Response to EP

Collective data indicate that the equine immune response is unable to eliminate *B. equi*, and infected horses are reported to remain infected for life (Schein, 1988; Rothschild and Knowles, 2007). Research for an effective treatment that eliminates or clears the horse of *B. equi* infection is underway. Although infections with *B. caballi* have been described as self-limiting, lasting up to 4 years after infection (de Waal and van Heerden, 2004), many horses that have apparently recovered from *B. caballi* infection relapse, suggesting lifelong infection and a period of time in which the pathogen was undetectable but still present. In addition, some scientists believe that the observations of *B. caballi* clearance were made before the development of more sensitive diagnostic methods, such as nested polymerase chain reaction (PCR) (Rothschild and Knowles, 2007). Additional research is warranted to determine whether *B. caballi* and *B. equi* infections can be eliminated and will rely on the development of a testing scheme that ensures the detection of any remaining pathogens in treated animals.

Resistance to clinical disease and re-infection in previously exposed animals has been described and is thought to be due to continued immune stimulation by the parasites, although the exact mechanisms of resistance have not been elucidated (Maurer, 1962; Rothschild and Knowles, 2007). Clinical relapses in carrier equids may be due to immune suppression, concurrent disease, or splenectomy (Maurer, 1962). There is no cross immunity between *B. caballi* and *B. equi* (Maurer, 1962; Taylor et al., 1969).

Persistent infection of equids is extremely important in the maintenance of EP in nature. Even though the number of EP pathogens in the erythrocytes of carrier equids is typically low, these animals act as reservoirs of EP pathogens and may serve as sources for dissemination of the pathogens wherever tick vectors or other transmission opportunities occur (Ueti et al., 2005; Rothschild and Knowles, 2007).

References—Equine Immune Response to EP

- de Waal DT, van Heerden J, 2004. Equine piroplasmiasis. In *Infectious Diseases of Livestock*, ed. Coetzer JAW. Oxford University Press, Capetown, South Africa.
- Maurer FD, 1962. Equine piroplasmiasis—another emerging disease. *J Am Vet Med Assoc* 141:699–702.
- Rothschild C, Knowles D, 2007. Equine piroplasmiasis. In *Equine Infectious Diseases*, ed. Sellon DC, Long Mt. Saunders, Elsevier, St. Louis, MO. 465–473.
- Schein E, 1988. Equine babesiosis. In *Babesiosis of Domestic Animals and Man*, ed. Ristic M. CRC Press, Boca Raton, FL. 197–208.
- Taylor WM, Bryant JE, Anderson JB, Wilkens KH, 1969. Equine piroplasmiasis in the United States—a review. *J Am Vet Med Assoc* 155:915–919.
- Ueti MW, et al., 2005. Ability of the vector tick *Boophilus microplus* to acquire and transmit *Babesia equi* following feeding on chronically infected horses with low-level parasitemia. *J Clin Microbiol* 43:3755–3759.

This test cannot evaluate sera with anticomplement activity (Friedhoff and Soulé, 1996). The CFT was first implemented as an import test for equids entering the United States in 1970 (Federal Register, 1979) and remained the official test until 2005. When testing horses with acute infections, CFTs have been positive, while other serologic tests, such as the cELISA, have been negative (Short, pers. comm., 2010). Disadvantages associated with the CFT include the need to produce large quantities of antigens and the occurrence of false-negative results (Brüning, 1996).

Indirect Immunofluorescent Antibody Test (IFAT)

The IFAT is a more sensitive test than the CFT and has been used as a supplemental test when CFT results are inconclusive (Rothschild and Knowles, 2007). In this assay, parasite antigens are bound to a glass slide and allowed to react with test sera. Bound antibodies are visible under ultraviolet light after binding of the fluorescein-labeled anti-equine sera. Sera are considered positive if they show strong fluorescence of the parasites at a dilution of 1:80 or higher. In one study, the earliest antibody responses detected with the IFAT in horses experimentally infected with *B. caballi* and *B. equi* occurred 3 to 20 days after infection, with titers still detected in the chronic stages of infection. To increase specificity with the IFAT, the serum must be diluted, resulting in a concurrent loss of sensitivity (Rothschild and Knowles, 2007). The IFAT is time consuming, requires large amounts of antigen and, because of the subjectivity in interpreting fluorescence, is difficult to standardize. Per the OIE manual, the IFAT is one of the prescribed tests for purposes of equine international trade (OIE, 2005).

Enzyme-linked Immunosorbent Assay (ELISA) and Competitive Inhibition ELISA

Knowles and others (1991), using *B. equi* equine merozoite antigen (EMA)-1 and specific monoclonal antibodies, developed a competitive inhibition ELISA (cELISA) to detect *B. equi* infection. This cELISA was later improved by using recombinant protein instead of culture-derived whole parasites (Knowles et al., 1992). The cELISA has detected chronic infection in experimentally infected horses not detected by the CFT (Brüning, 1996). The use of the recombinant protein facilitates standardization of the assay and overcomes the need for in vitro cultivation of the parasite or the artificial infection of horses for antigen production, making the cELISA an ideal screening test for *Babesia* infection (Rothschild and Knowles, 2007).

In 1999, a cELISA using recombinant *B. caballi* rhoptry-associated protein-1 (RAP-1) was developed by Kappmeyer and others (1999). In a field survey, this test identified 25 percent more sera as positive for *B. caballi* than did the CFT.

In 2004, the OIE approved the cELISA—for both *B. equi* and *B. caballi* detection—as a prescribed test for international horse movement (OIE, 2005; Rothschild and Knowles, 2007). The NVSL's "Competitive ELISA for Serodiagnosis of Equine Piroplasmiasis (*Babesia equi* and *B. caballi*) and Production of Recombinant *B. equi* and *Babesia caballi* cELISA Antigens" is the cited reference for cELISA EP test methods in the OIE's "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals" (OIE, 2005). Subsequently, two test kits manufactured by Veterinary Medical Research and Development (VMRD) were developed—one for detecting *B. equi* antibodies and one for detecting *B. caballi* antibodies. These kits are licensed by the USDA-APHIS-VS Centers for Veterinary Biologics (CVB) based on performance requirements for the kits and each batch of reagents. The sale of these licensed cELISA test kits in the United States is restricted to specific USDA-approved laboratories.

Approval of test kits for use in the United States

The VMRD cELISA test for *B. caballi* and *B. equi* are listed as licensed kits by CVB on its Web site (USDA, 2010). In another section of the Web site there is a remark that diagnostic test kits for diseases with U.S. State and/or Federal eradication/control programs will be provided to the NVSL by CVB for evaluation for program use. In some cases, APHIS may provide the core samples for a reference serum panel. The license and/or permit for such kits may restrict distribution to APHIS-approved laboratories.

Western blot

The Western blot test is also called the immunoblot assay or Iblot. The materials and methods for this test have been described by Schwint and others (2009), and thus far the test has been used primarily in research. When the Iblot was compared to CFT and cELISA in two horses treated for *B. caballi* infection with imidocarb dipropionate, the Iblot test remained positive longer than either the CFT or cELISA. However, in a serosurvey conducted by USDA-APHIS-VS the confirmatory testing of VMRD cELISA-positive samples via Western blot did not lead to usable results due to several factors, including inadequate remaining sera from some of the samples, poor sera quality, and unexpected results (banding patterns) when Western blot testing these field samples. These results were in contrast to those from limited experimental use of the Western blot in which clear indication for positive and negative horses was provided (USDA, 2009).

Polymerase chain reaction (PCR)

The PCR test relies on amplification of DNA from the EP pathogens and the detection of specific, unique components of that DNA. There are four different types of PCR test used for detecting *Babesia* parasites: conventional or primary PCR (one set of primers); real-time PCR (quantifies level of parasite in peripheral blood); nested PCR (two sets of primers used to increase sensitivity); and nested PCR with hybridization (probe specific for gene target results in enhanced sensitivity and specificity) [D. Knowles, pers. comm., 2010]. It is important to note that specificity in the PCR can be defined by several methods. These include molecular mass of the band, restriction endonuclease treatment of the band, sequencing of the band, and hybridization with specific probes (D. Knowles, pers. comm., 2010). Therefore, under experimental conditions false positives can be avoided. A real-time PCR test that allows for quantitative detection of *B. caballi* and *B. equi* has been reported (Bhoora, 2010). Quantification of the parasite in blood would allow for studies related to factors that may alter the level of parasitemia.

A nested PCR for *B. equi* based on the sequence of the EMA-1 gene has detected the equivalent calculated parasitemia of 0.000006 percent (Nicolaiewsky et al., 2001). In a field study, this nested PCR detected 3.6 times more infections than did microscopy and 2.2 times more infections than did conventional PCR (Rothschild and Knowles, 2007).

To assess the diagnostic sensitivity and specificity of the PCR, studies that include testing horses in endemic areas are necessary. In addition, there is a need to define the transmission risk posed by a seropositive, but PCR-negative, horse in order to define the horse's status from a transmission and regulatory perspective. Currently, the PCR is used only for research purposes in the United States; use of the test for regulatory purposes to determine a horse's infection status will not be considered until further validation of the tests is completed.

Animal inoculation

The OIE Terrestrial Animal Health Manual indicates that when equivocal results are encountered in serological tests for EP, the inoculation of large quantities of whole blood transfused from the suspect horse into a susceptible splenectomized horse will assist in diagnosis (OIE, 2008). The recipient horse is observed for clinical signs of disease, and its red blood cells are examined for parasites. Alternatively, a specific tick vector is fed on the suspect animal and the parasite may then be identified either in the vector or through transmission by the vector to another susceptible animal. Transmissions by direct blood inoculation and by tick feeding were used to demonstrate the clearance of *B. caballi* based on treatment with imidocarb dipropionate (Schwint et al., 2009).

Reporting criteria for National Animal Health Reporting System (NAHRS)

Equine piroplasmiasis is considered a foreign animal disease in the United States. Accredited veterinarians are required to report all animal disease conditions to regulatory veterinary authorities if the differential diagnosis could include a foreign animal disease. A USDA-certified Foreign Animal Disease Diagnostician is assigned to investigate using standardized protocol and submits diagnostic materials to

the NVSL. Reporting through NAHRS requires the concurrence of the USDA and the State Chief Animal Health Official. Diagnosis requires serologic confirmation (CF) at the NVSL, an epidemiologic investigation, and concurrence of the State Chief Animal Health Official and the Federal Area Veterinarian in Charge (NAHRS, 2008).

Laboratory Criteria: OIE Manual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals 2008, Chapter 2.5.8 (OIE, 2005)

- 1.1. *Agent identification:* Geimsa stained blood or organ smears taken during the acute phase of the disease can be used to identify the parasites, but this method may be quite difficult in samples from chronically infected carrier animals. Thick blood film samples may help in chronic infections. Polymerase chain reaction (PCR) tests have been developed.
- 1.2. *Serological tests:* Serum is tested for the presence of antibodies by complement fixation (CF), or indirect fluorescent antibody (IFA), or competitive enzyme-linked immunosorbent assays (c-ELISA). Positive CF and IFA results indicate a more acute infection and start to wane by 180 days. cELISA positive results develop at approximately three weeks and indicate a more chronic infection.
2. **Case Definition:**
 - 2.1. *Suspect case:* Any equids with:
 - 2.1.1. Clinical signs consistent with equine piroplasmosis; **OR**
 - 2.1.2. History of exposure; **OR**
 - 2.1.3. An inconclusive or positive test (Geimsa-stained blood film, or CF, or IFA, or c-ELISA, or PCR) performed on sample taken during routine screening or surveillance for equine piroplasmosis.
 - 2.2. *Presumptive positive case:* Any suspect case with:
 - 2.2.1. Epidemiological information consistent with equine piroplasmosis; **AND**
 - 2.2.2. A positive test for equine piroplasmosis (Geimsa-stained blood film, or CF, or IFA, or c-ELISA, or PCR).
 - 2.3. *Confirmed positive case:* Any equids tested confirmed positive for equine piroplasmosis by **NVSL or a laboratory designated by the Secretary of Agriculture** by at least one of the following methods: CF **OR** c-ELISA.
3. **Reporting Criteria:** Equine piroplasmosis is a U.S. foreign animal disease (FAD) outside Puerto Rico and the U.S. Virgin Islands, and is an OIE notifiable disease.
 - 3.1. Follow standard FAD procedures according to Veterinary Services Memorandum No. 580.4.
 - 3.2. Any suspect cases must, by law, be reported immediately to State Veterinary Officer or Federal authorities (AVIC).

References—Diagnostic Testing for EP

- Bhoora R, Quan M, Franssen L, Butler CM, van der Kolk JH, Guthrie AJ, Zwegarth E, Jongejan F, Collins NE, 2010. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. *Vet Parasitol* 168(3/4):201–211.
- Brüning A, 1996. Equine piroplasmosis: an update on diagnosis, treatment and prevention. *Br Vet J* 152(2):139–151.
- Donnellan CMB, Marais HJ, 2009. Equine piroplasmosis. In *Infectious Diseases of the Horse*, ed. Mair TS, Hutchinson RE, Equine Veterinary Journal, Inc. 333–340.
- Federal Register, 1979. Proposed Rule 9 CFR Part 92, April 27, 1979. 44(83):24863-24864.
- Friedhoff KT, Soulé C, 1996. An account on equine babesiosis. *Rev Sci Tech Off Int Epiz* 15(3):1191–1201.
- Kappmeyer LS, Perryman LE, Hines SA, Baszler TV, Katz JB, Hennager SG, Knowles DP, 1999. Detection of equine antibodies to *Babesia caballi* by recombinant *B. caballi* rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. *J Clin Microbiol* 37(7):2285–2290.
- Knowles DP, Perryman LE, Kappmeyer LS, Hennager SG, 1991. Detection of equine antibody to *Babesia equi* merozoite proteins by monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. *J Clin Microbiol* 29(9):2056–2058.

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- Knowles DP, Kappmeyer LS, Stiller D, Hennager SG, Perryman LE, 1992. Antibody to recombinant merozoite protein epitope identifies horses infected with *B. equi*. *J Clin Microbiol* 30(12):3122–3126.
- Nicolaiewsky TB, Richter MF, Lunge VR, Cunha CW, Delagostin O, Iluta N, Fonseca AS, da Silva SS, Ozaki LS, 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. *Vet Parasitol* 101(1):9–21.
- OIE, 2005. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, ch. 2.5.6 Equine Piroplasmosis. Available at: http://www.oie.int/eng/normes/mmanual/A_00084.htm
- Rothschild C, Knowles D, 2007. Equine piroplasmosis. In *Equine Infectious Diseases*, ed. Sellon DC, Long MT. Saunders, Elsevier, St. Louis, MO. 465–473.
- Schwint ON, Uiti MW, Palmer GH, Kappmeyer LS, Hines Mt, Cordes RT, Knowles DP, Scoles GA, 2009. Imidocarb dipropionate clears persistent *Babesia caballi* infection with elimination of transmission potential. *Antimicrob Agents and Chemother* 53:4327–4332.
- Sippel WL, Cooperrider DE, Gainer JH, Allen RW, Mouw JEB, Tiegland MB, 1962. Equine piroplasmosis in the United States. *J Am Vet Med Assoc* 141:694–698.
- Theiler A, 1902. Equine malaria. *J Comp Path and Therap* 15:40–54.
- USDA—APHIS, 2009. Seroprevalence of equine piroplasmosis disease agents in the United States. #569.1009. Available at: <http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/equine/EP/%20info%20sheet.pdf>
- USDA—Center for Veterinary Biologics, 2010a. Licensed Biologics. Available at: http://www.aphis.usda.gov/animal_health/vet_biologics/
- Veterinary Medical Research and Development (VMRD), 2005. VMRD Newsletter, April 2005. Available at: http://www.vmr.com/docs/nl/04_2005nl.pdf
- Veterinary Medical Research and Development (VMRD), 2006. VMRD Newsletter. Available at: <http://www.vmr.com/docs/catalogs/2006-Int.pdf>

Treatment of Equids with EP

Treatment regimens for equids with EP often vary and may depend on whether the equids are in endemic or free countries, and whether the desired goal of treatment is parasite elimination or resolution of clinical disease.

Treatment of EP in endemic countries is usually aimed at eliminating clinical signs of disease without eliminating the parasite from the body. It is detrimental to eliminate the parasite from equids in endemic countries because premunition depends on continued presence of the parasite at low levels (Donnellan and Marais, 2009).

In contrast, in countries where EP is not considered endemic, treatment of infected equids is aimed at chemosterilization. Clearing the parasites by treating infected equids with antiprotozoal drugs is challenging and treated horses may continue to be reservoirs. These pathogens may persist in immunocompetent hosts at levels below the limits of routine microscopic detection and without overt signs of disease, yet serve as efficient reservoirs of arthropod vector-borne transmission (Ueti et al., 2008; Schwint et al., 2009). Defining the criteria to determine if parasite clearance has occurred is also a challenge, as the goal is to be confident that the treatment has eliminated the transmission risk. At this time, treating infected equids in the United States is not authorized, unless the horse is enrolled in a USDA research trial.

Antiparasitic drugs

A variety of drugs have been used in attempts to treat EP. In general, *B. equi* has been reported to be more refractory to babesiacidal drugs than *B. caballi*. The drugs commonly used to treat EP in equids are imidocarb and diminazene.

Historically, amicarbalide isothionate and eufalvine were used to treat EP, but these drugs are no longer readily available (Donnellan and Marais, 2009). Tetracycline has been reported as a treatment for *B. equi*; however, in these cases the drug was given prior to definitive identification of the causative agent, so treatment response in suspect cases might have been the result of resolution of a different type of infection (Zobba, 2008). Antitheilerial compounds, including parvaquone and buparvaquone, have been shown to reduce parasitemia of initial infection but failed to sterilize *B. equi* infections. Imidocarb appears to be the most effective of the drugs explored to date (Donnellan and Marais, 2009).

Imidocarb

Imidocarb is a caranilide derivative usually administered as the dipropionate salt by intramuscular injection. Although imidocarb has been used for more than 20 years to treat babesiosis, minimal information was available about the pharmacokinetic behavior of the drug in equids until 2002, when Belloli and others (2002) published an article on the topic. Belloli's study showed that: (1) imidocarb was effectively distributed to tissues; (2) a prolonged period was required for complete elimination of the drug from tissues; and (3) there was evidence of possible sequestration of imidocarb in vascular and extravascular compartments.

The short-lasting persistence of imidocarb in blood reported by Belloli was not considered a favorable kinetic behavior for a drug to affect hemoprotozoan parasites. Reports of the plasma concentration of imidocarb necessary for effective therapy are lacking for equine *Babesia* spp. Based on the effective diffusion of imidocarb across cell membranes, the short-lasting persistence of the drug in blood may be enough to kill *B. caballi* (Belloli et al., 2002).

The liver was reported to be a storage tissue for imidocarb. In Belloli's study, imidocarb was detected in the milk of mares, but mammary elimination did not appear to be important. The concentration of imidocarb in milk reflected the state of the plasma drug levels (Belloli et al., 2002). Given the rapid clearance of imidocarb from plasma, repeating multiple courses of treatment with several days in between

may ensure that high drug levels are maintained in storage tissues, thereby supplying the body with delivery reservoirs from continuous release of imidocarb. The storage tissues (liver and kidney) may also be sites in which circulating infected cells are exposed repeatedly to elevated drug concentrations (Belloli et al., 2002).

Research results regarding the efficacy of imidocarb for *B. caballi* and *B. equi* clearance vary. Schwint and others (2009) experimentally inoculated horses with a Puerto Rican strain of *B. caballi* then treated them with imidocarb dipropionate (Imizol, Schering Plough Animal Health) via intramuscular injection. Treated horses failed to transmit infection to naïve horses by transfer of whole blood or by tick feeding. The treated horses converted from seropositive to seronegative status on the cELISA test. The authors concluded that the aggressive imidocarb treatment cleared the strain of *B. caballi* inoculated in this study from persistently infected horses. Treated horses met the regulatory standard for international movement (seronegative on cELISA test) and eliminated the transmission risk to ticks.

Paso Fino horses imported to the island of Curacao that were seropositive to *B. caballi* and *B. equi*—based on testing with the indirect fluorescent antibody test (IFAT)—were treated with five consecutive doses of imidocarb dipropionate. This treatment protocol temporarily resulted in negative serologic tests; however, samples collected 6 and 18 weeks after treatment tested positive for both *B. caballi* and *B. equi* antibodies (Butler et al., 2008).

Ongoing research at the NVSL is evaluating the efficacy of imidocarb dipropionate in the clearance of *B. equi* from experimentally infected horses. Dr. Tom Bunn, Director of Diagnostic Bacteriology Laboratory at the NVSL, reported that the goal of this research is to attempt to demonstrate the ability of imidocarb to clear horses infected with *B. equi* (USAHA, 2009). Twelve ponies were inoculated with *B. equi*, 8 of which were subsequently treated with imidocarb. Clearance was defined by a negative PCR test, the failure to establish infection by direct inoculation of blood to susceptible recipient horses, and the failure of ticks to acquire parasites after feeding from treated ponies. At the time of this report, three naïve horses had been inoculated with red blood cells (RBCs) from treated, PCR-negative ponies and showed no seroconversions after 90 days (USAHA, 2009). Two naïve intact horses inoculated with RBCs from untreated ponies seroconverted by both CFT and cELISA after 90 days. The next steps in the project are to continue subinoculation of seronegative horses (USAHA, 2009). If blood inoculation of horses fails to result in seroconversion, ticks that have fed on treated horses will be fed on susceptible horses to determine if *B. equi* is transmitted (USAHA, 2009). As a final test of clearance, splenectomies will be performed.

Imidocarb causes a dose-dependent hepatotoxicity and nephrotoxicity (Donnellan and Marais, 2009). In clinical cases with dehydration and anemia drug disposition kinetics may potentiate the negative effects of imidocarb. The LD50 for an imidocarb injection given intramuscularly was 15.99 ± 1.49 mg/kg, with mortalities occurring within 6 days following the first injection. Increasing levels of imidocarb was associated with increasing mortality and morbidity (local and systemic reactions). Mortalities were attributed to acute renal cortical tubular necrosis and acute periportal hepatic necrosis induced by two injections of 16 or 32 mg/kg of imidocarb (Adams, 1981).

Treatment of donkeys with EP

Donkeys are reported to be more susceptible than horses to the toxic effects of imidocarb (Donnellan and Marais, 2009). The therapeutic efficacy of imidocarb, artesunate, arteether, buparvaquone, and a combination of arteether and buparvaquone against *B. equi* of Indian origin was evaluated in splenectomized donkeys with experimentally induced acute infection (Kumar et al., 2003). Individually, arteether and buparvaquone had no efficacy for clearing parasitemia; treated donkeys died within 5 to 6 days after showing high parasitemia and clinical signs of disease. Parasite multiplication was restricted in donkeys treated with artesunate, but only while drugs were given. Donkeys treated with imidocarb and a combination of arteether and buparvaquone cleared *B. equi* from blood circulation 2 to 5 days post treatment; however, reoccurrence of the parasite was detected in both groups 55 to 58 days post treatment.

In vitro testing³

The literature contains information about in vitro effects of compounds on EP agents and their relation to the survival of *B. caballi* and *B. equi*. Chemicals or drugs that kill the parasites in the laboratory may not have the same effect when given to equids and may in fact be toxic to equids. Before a drug is used in equids, stringent evaluation of its efficacy and safety is usually undertaken by the manufacturer. However, the costs associated with the research, development, and licensing of a new drug can be substantial, and a manufacturer will not make such an investment unless a market exists for the drug.

Evaluating the effect of antimicrobial drugs in an in vitro model has utility in screening antiparasitic drugs for potential in vivo use. As an example of a model, the effect of oxytetracycline, imidocarb, and enrofloxacin on *A. marginale*, a hemoparasite of cattle, was recently evaluated using short-term erythrocyte cultures (Coetzee et al., 2009). In this evaluation, a significant association existed between ultrastructural changes in the parasite and infectivity.

In vitro growth inhibition of *B. equi* and *B. caballi* was reportedly induced by exposure to triclosan, with no adverse effects on host cells (Bork et al., 2003). The multiplication of *B. equi* and *B. caballi* in an in vitro cell culture model was significantly inhibited by heparin, resulting in complete clearance of the intracellular parasites (Bork et al., 2004). Growth inhibition of *B. equi* and *B. caballi* in vitro was reported due to exposure to three antimalarial drugs, including artesunate, pyrimethamine, and pamaquine (Nagai et al., 2003). *B. equi* was more resistant to pyrimethamine than *B. caballi* and more sensitive to artesunate and pamaquine than *B. caballi*.

A *B. caballi* culture system has been used to evaluate the in vitro antibabesial efficacy of four ethnoveterinary plants: *Rhoiscissus tridentate*, *Elephantorrhiza elephantina*, *Aloe marlothii*, and *Urginea sanguinea* in vitro (Naidoo et al., 2005). To validate the model, the plants were compared with imidocarb dipropionate and diminazene aceturate. Effectivity was established as the degree of inhibition of color change, the percentage of parasitized cells on thin culture smears, and calculation of the degree of residual infectivity. The model demonstrated the in vitro efficacy of imidocarb and diminazene indicating EC50 values of 0.08 and 0.3 µg/ml, respectively. Of the ethnoveterinary plants, only the *E. elephantina* rhizomes acetone extracts at a concentration of 100 µg/ml were effective. This study showed that the color change method was not very sensitive in determining the activity of crude plant extracts (Naidoo et al, 2005).

Additional treatment and management

Antibabesial treatment should be accompanied by supportive care of horses with clinical disease. Supportive care would involve management of anemia through blood transfusion and fluid treatment in animals with pigmenturia or dehydration.

Equine piroplasmosis is a reportable disease in the United States, and veterinarians should contact State or Federal regulatory officials rather than treat suspect cases.

References—Treatment of Equids with EP

- Adams LG, 1981. Clinicopathological aspects of imidocarb dipropionate toxicity in horses. *Res in Vet Sci* 31:54–61.
- Belloli C, Crescenzo G, Lai O, Carofigliio V, Marang O, Ormas P, 2002. Pharmacokinetics of imidocarb dipropionate in horses after intramuscular injection. *Eq Vet J* 34:625–629.
- Bork S, Yokoyama N, Ikehara Y, Kumar S, Sugimoto C, Igarashi I, 2004. Growth inhibitory effect of heparin on Babesia parasites. *Antimic Agents and Chemoth* 48:236–241.
- Bork S, Yokoyama N, Matsuo T, Glavera GG, Fujisaki K, Igarashi I, 2003. Growth inhibitory effect of triclosan on equine and bovine Babesia parasites. *Am J Trop Med and Hyg* 68:334–340.

³ In vitro information included to provide a complete account of described drugs/substances and their effect on EP pathogens.

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- Butler CM, Nighof AM, van der Kolk JH, Haseth Ob de, Taoufik A, Jongehan F, Houwers DJ, 2008. Repeated high dose imidocarb dipropionate treatment did not eliminate *B. caballi* from naturally infected horses as determined by PCR-reverse line blot hybridization. *Vet Parasitol* 151:320–322.
- Coetzee JF, Kocan KM, Higgins JJ, Apley MD, Jones DE, 2009. Ultrastructural and fluorochromatic changes of *Anaplasma marginale* exposed to oxytetracycline, imidocarb and *enrofloxacin* in short-term erythrocyte cultures. *Vet Microbiol* 136(1/2):45–53.
- Donnellan CMB, Marais HJ, 2009. Equine piroplasmosis. In *Infectious Diseases of the Horse*, ed. Mair TS, Hutchinson RE, Equine Veterinary Journal, Inc. 333–340.
- Kumar S, Kumar Y, Malhotra DV, Dhar S, Nichani AK, 2003. Standardisation and comparison of serial dilution and single dilution enzyme linked immunosorbent assay (ELISA) using different antigenic preparations of the *Babesia (Theileria) equi* parasite. *Vet Parasitol* 34(1):71–83.
- Nagai A, Yokoyama N, Matsuo T, Bork S, Hirata H, Xuan X, Zhu Y, Claveria G, Fujisaki K, Igarashi I, 2003. Growth inhibitory effects of artesunate, pyrimethamine and pamaquine against *Babesia equi* and *Babesia caballi* in *in vitro* cultures. *Antimicro Agents and Chemoth* 47.
- Schwint ON, Uiti MW, Palmer GH, Kappmeyer LS, Hines Mt, Cordes RT, Knowles DP, Scoles GA, 2009. Imidocarb dipropionate clears persistent *Babesia caballi* infection with elimination of transmission potential. *Antimicro Agents and Chemoth* 53:4327–4332.
- Ueti MW, Palmer GH, Scoles GA, Kappmeyer LS, Knowles DP, 2008. Persistently infected horses are reservoirs for intrastadial tick-borne transmission of the apicomplexan parasite *Babesia equi*. *Infect Immun* 76:3525–3529.
- USAHA, 2009. ICOHC proceedings. Available at: <http://www.usaha.org/committees/reports/2009/report-hd-2009.pdf>
- Zobba R, Ardu M, Nicconlini S, Chessa B, Manna L, Cocco R, Parpaglia MLP, 2008. Clinical and laboratory findings in equine piroplasmosis. *J Eq Vet Sci* 28(5):301–308.

Distribution, Biology, and Ecology of U.S. EP Tick Vectors (revised 11/10/10)

Ticks are commonly thought of as insects but are actually arachnids like scorpions, spiders, and mites. All members of this group have four pairs of legs as adults and have no antennae. Adult insects have three pairs of legs and one pair of antennae. Ticks are among the most efficient carriers of disease agents because they attach firmly to a host when sucking blood, feed slowly, and may go unnoticed for a considerable time while feeding. There are at least 850 tick species worldwide and approximately 85 tick species within the United States (Horak et al., 2002).

Life stages/life cycle

Ticks are divided into two groups: hard ticks and soft ticks. Hard ticks such as the American dog tick have a hard shield just behind the mouth parts (sometimes incorrectly called the "head"). Unfed hard ticks are shaped like a flat seed. Soft ticks do not have the hard shield and are shaped like a large raisin. Soft ticks prefer to feed on birds or bats and are seldom encountered by people or other types of animals unless their host animals are nesting or roosting in an occupied building.

Ticks have four life stages: egg, six-legged larva, eight-legged nymph and adult. After the egg hatches, the tiny larva feeds on an appropriate host. The larva then develops (molts) into the larger nymph. The nymph feeds on a host and then molts into an even larger adult. Both male and female adults find and feed on a host, the females lay eggs after feeding (Sonenshine, 1991). Female adult ticks die after laying eggs. Male adult ticks can feed and mate multiple times before dying, and their life span varies, depending on species. When seeking a female for mating, male ticks might move from one animal host to another.

Ticks are generally classified as one-, two-, or three-host ticks.

One-host tick

A one-host tick attaches to a single host and feeds on that same host throughout all of its life stages, from larva through adult. A one-host tick attaches to a host at the larval stage, feeds, molts to the nymphal stage on the host, feeds again on the same host, molts to an adult stage on the host, and feeds on the same host. The female will drop from the host after feeding and lay her eggs in a suitable habitat.

Two-host tick

A two-host tick attaches and feeds on at least two different hosts during its life stages. Generally, the immature stages feed on one host and the adult feeds on another.

Three-host tick

As a larva, a three-host tick will stay on the ground or climb up a plant to wait for a host to pass by. The seed tick (a larval form of a tick, which is very small) waits with its front claws outstretched to grab the first animal that comes by, usually a mouse, vole, squirrel, chipmunk, mole, shrew, muskrat, or rabbit (first host). The larva engorges with blood for about 4 days then drops to the ground, molting (shedding its skin) in leaf litter and becoming an eight-legged nymph. The nymph climbs up a plant and waits for a new host (second), which may be another small mammal or a moderate-sized animal like an opossum or raccoon. Once the nymph has attached to a new host, it engorges with blood for approximately 6 days then drops from its second host and molts on the ground. At this stage, it may take weeks before the nymph molts and becomes an adult tick.

As adults, both the male and female ticks look for a host (third in life cycle), most likely a larger mammal than its first or second host such as a raccoon, opossum, fox, skunk, woodchuck, deer, dog, or human. Once attached to a host, the female will feed to produce eggs. The male mates with the female while she completes her blood meal. A female will not complete her feeding period without mating. Rather, she will

remain on the host with a partial blood meal until she mates. After mating, the male dies and the female drops to the ground and lays her eggs, which begins the cycle again.

During any of its stages, a three-host tick can survive for several years without finding a host. In such cases, a tick may enter a diapause or a period of inactivity or rest, during which development is suspended (Oliver, 1989). Diapause is an adaptation by ticks used to survive unsuitable conditions. Ticks wait for host animals from the tips of grasses and shrubs (not from trees). When brushed by a moving animal or person, they quickly let go of the vegetation and climb onto the host. Ticks can only crawl; they cannot fly or jump. Some species of ticks will crawl several feet toward a host (Sonenshine, 1991).

Transmission and vector competency for EP pathogens

There are three main modes of tick-borne transmission of *Babesia* parasites: transstadial, intrastadial, and transovarial. Transstadial transmission occurs when a tick stage (larval or nymphal) acquires the pathogen from an infected host and the subsequent tick stage within the same generation transmits the pathogen to an uninfected host. The pathogen is retained by the tick through the molting process (i.e., nymph to adult). Intrastadial transmission occurs when a tick acquires the pathogen (i.e., male) transmits to a naïve host with no development or molting to another life stage prior to transmission. Transovarial transmission is the passage of the pathogen from one tick generation to the next through the ovaries (Ueti et al., 2008).

EP pathogens are enzootic on several islands in the Caribbean, areas in Central and South America, and in Mexico, and are transmitted by three genera of ticks, including *Hyalomma*, *Dermacentor*, and *Rhipicephalus* species (de Waal, 1992; Strickland and Gerrish, 1964). *B. caballi* is transmitted by seven *Dermacentor* species, six *Hyalomma* species, and two *Rhipicephalus* species. *B. equi* is transmitted by four *Dermacentor* species, four *Hyalomma* species, and five *Rhipicephalus* species. *B. caballi* is transmitted transstadially (nymph to adult) by 10 tick species and transovarially (female to eggs) by 11 tick species. *B. equi* is transmitted transstadially by 13 tick species and only transovarially by *H. anatolicum* (Neitz, 1956; de Waal, 1992; Stiller and Coan, 1995). *Anocentor nitens*, the tropical horse tick, is currently the only known natural vector of EP in the United States (Roby and Anthony, 1963). *B. caballi* and *B. equi* have been shown to be experimentally transmitted by three additional U.S. tick species: *D. albipictus*, the winter tick; *D. variabilis*, the American dog tick; and *B. microplus*, the southern or tropical cattle tick. *B. caballi* can be transmitted by *A. nitens*, *D. albipictus*, and *D. variabilis*, whereas *B. equi* is transmitted only by *D. variabilis* and *Rhipicephalus (Boophilus) microplus* (Stiller et al., 2002). Although *R. microplus* has been considered an experimental vector of *B. equi*, evidence is growing in Brazil that *R. microplus* is likely a natural vector of *B. equi* in Brazil (Guimaraes et al., 1998; Heuchert et al., 1999; Battsetseg et al., 2002). *B. equi* infects horses and is transmitted by *R. microplus* in subtropical and tropical regions of the Americas (Knowles et al., 1992; Guimaraes et al., 1998). Transstadial transmission of *B. equi* by *R. microplus* has been confirmed with the acquisition of parasites by the nymphal stage from chronically infected horses and transmitting as a newly molted adult to a naïve host (Ueti et al., 2005). Additionally, *R. microplus* males can acquire *B. equi* parasites from a chronically infected horse and transmit the parasites to a naïve horse. Therefore, intrastadial transmission has been demonstrated with this tick vector, and targeted control methods should consider this mechanism of transmission (Ueti et al., 2008). Additionally, there is some recent evidence that *D. variabilis* and *Amblyomma cajennense*, the cayenne tick, may be natural vectors of *B. equi*, as demonstrated with the field collection of adults and transmission of *B. equi* to naïve horses (Scoles, pers. comm., 2010). It is likely that there is some intrastadial transmission of *B. equi* (same tick stage, i.e., males) by American dog tick and cayenne tick populations to naïve hosts (Stiller and Coan, 1995; Scoles, pers. comm., 2010).

Distribution, host associations, and seasonal activity

The major factors involved in the occurrence of a vector-borne disease include: the abundance of vectors and reservoir hosts; prevalence of pathogens within vectors and vertebrate hosts; local environment conditions, particularly temperature and moisture for tick vectors; and host resistance in the targeted host population (Sonenshine, 1991).

Natural vectors are arthropods capable of transmitting pathogens under essential environmental conditions whereby the pathogens survive and multiply within the vector and the pathogen is transmitted to a naïve host. The vector is capable of obtaining the pathogen via intake of a large blood meal from an infective host and maintaining the pathogen through multiple life stages. An experimental vector is defined as an arthropod that is infected via an inoculated host in a laboratory setting and that can maintain and transmit the pathogen to naïve host under artificial conditions (Sonenshine, 1991). A tick species is considered established when it is capable of reproducing successfully (i.e., completes its life cycle) under favorable abiotic and biotic conditions in a particular region. If the tick species is considered "reported" from an area, it is likely that the success of reproductive status of that particular tick species has not been confirmed and the tick(s) might simply have been imported on a host (Dennis et al., 1998).

Natural and experimental EP tick vectors in the United States

Natural vectors

Tropical horse tick (Anocentor nitens)

A natural vector of *B. caballi* in the Americas is the tropical horse tick *Anocentor nitens* (also known as *Dermacentor nitens*) (Horak et al., 2002; Barker and Murrell, 2004); its distribution in the United States is limited to the southernmost parts of Florida and Texas. The tick is widespread in the tropical and subtropical regions of northern South America, Central America, Mexico, and the West Indies. In 1901, *A. nitens* distribution extended from Jamaica and Santo Domingo to include Puerto Rico, Guatemala, and Venezuela. By 1964, *A. nitens* distribution extended to the Caribbean, Central America, Mexico, and South America. The first report of *A. nitens* in the United States was in 1908 in Cameron County, Texas, with additional reports coming from Hidalgo, Nueces, San Patricio, Webb, and Willacy Counties by 1964. *A. nitens* was known to be established in the southernmost tip of Texas until 1960, when it was found in Palm Beach, Florida (Friedhoff et al., 1990). From 1960 through September 1963, there were 705 collections of *A. nitens* from 24 counties in Florida (Strickland and Gerrish, 1964). In 1962, *A. nitens* was collected from Coffee County, Georgia, on four separate occasions, but it did not become established in that State. From 1962 to 1963, collections of *A. nitens* were only made in Texas, Florida, and Georgia (Knowles et al., 1966; Bryant et al., 1969; Taylor et al., 1969). *A. nitens* submissions to NVSL from 1999 to 2008 are currently reported only from southern Texas (figure 1).

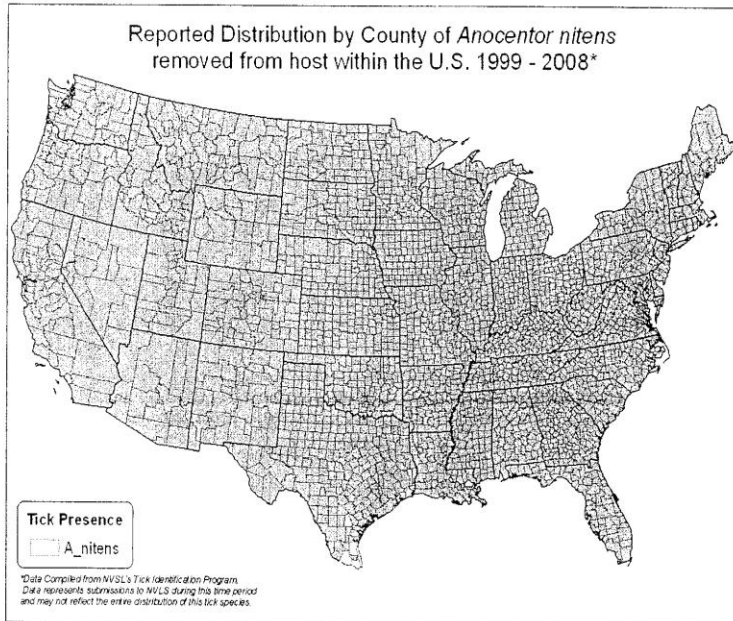


Figure 1

Horses, donkeys, mules, and other equids are the preferred hosts of *A. nitens*; however, this tick is also found on cattle, goats, sheep, and deer (Thompson, 1969). *A. nitens* is widespread in Brazil (Borges et al., 1999). It is typically found in the nasal diverticulum, the mane, perineal and perianal regions, and particularly the ears of the host. Heavy infestations of this tick can cause severe lesions and predispose a host to secondary infections.

As a one-host tick, *A. nitens* remains on its host through two molts and requires approximately 26 days to complete its development; it produces new generations at 75- to 90-day intervals. This extended development on the host provides opportunity for the tick to travel long distances (Bishopp and Trembley, 1945). This tick is found in all stages on hosts throughout the year (Holbrook et al., 1968; Borges et al., 2002).

Cattle tick (Rhipicephalus [Boophilus] microplus)

Although *B. microplus*, the southern or tropical cattle tick, was recently reclassified into the genus *Rhipicephalus*, for purposes of clarity, we will maintain the previous genus assignment of *Boophilus* (Keirans and Durden, 2001; Murrell and Barker, 2003). *B. microplus* is considered a natural vector of EP in South America. Neither *B. equi* nor *B. caballi* has been detected in field collections of *B. microplus* in the United States.

B. microplus is found worldwide in subtropical and tropical regions. This tick is endemic in the Indian subcontinent, much of tropical and subtropical Asia, northeastern Australia, Madagascar, southeastern Africa, the Caribbean, and many countries in South and Central America and Mexico. In 1906, *B. microplus* was well established within 14 southern U.S. States and southern California. By 1943, *B. microplus* was eradicated from all southern States except Florida, which successfully eradicated the tick by 1961. Although the tick has been eradicated from most of the United States, it can sometimes still be

found in Texas in a buffer quarantine zone along the Mexican border (Estrada-Pena, 2006) (figure 2). *B. microplus* is considered the most important tick of livestock in the world, based on economic impact and animal health. Heavy tick burdens on animals can decrease production and damage hides (Utech et al., 1983; Estrada-Pena, 2006). This hard tick can be found on many hosts including cattle, buffalo, horses, donkeys, goats, sheep, deer, pigs, dogs, and some wild animals.

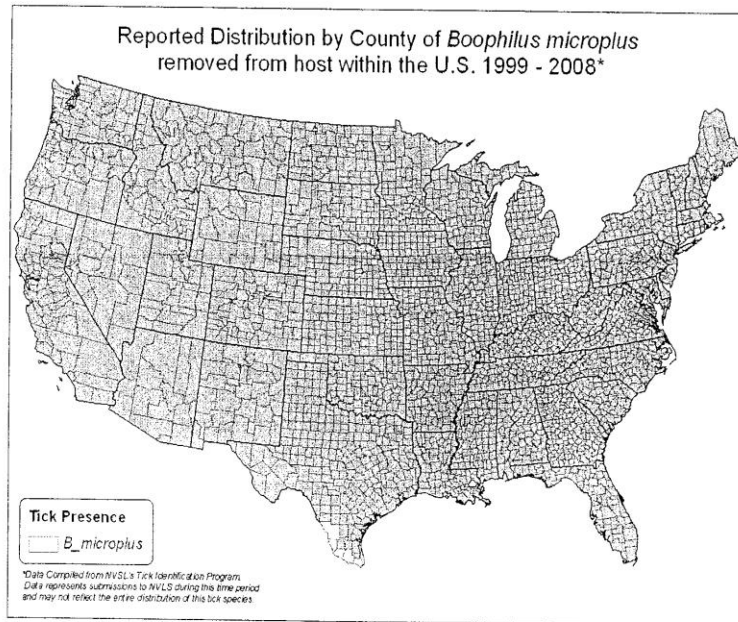


Figure 2

B. microplus is a one-host tick; all stages are spent on one animal. Its eggs hatch in the environment and the larvae crawl up grass or other plants to find a host. They may also be blown by the wind. In the summer, the *B. microplus* can survive for as long as 3 to 4 months without feeding, and under optimal conditions it completes its life cycle in 3 to 4 weeks. In cooler temperatures, the tick may live without food for up to 6 months. Newly attached tick larvae are usually found on the softer skin of the inner thigh, flanks, and forelegs of the host. They may also be found on the abdomen and brisket. After feeding, the larvae molt twice to become nymphs and then adults. Each developmental stage (larva, nymph, and adult) feeds only once, but the feeding takes place over several days. An adult male tick becomes sexually mature after feeding and mates with feeding females. An adult female tick that has fed and mated detaches from the host and deposits a single batch of many eggs in the environment (Sutherst et al., 1988; Guglielmo et al., 1990; Estrada-Pena, 2006).

Cayenne tick (Amblyomma cajennense)

A. cajennense, the cayenne tick, is a native of the Americas. *A. cajennense* ranges from the southern part of the United States to northern Argentina, through Central America and some of the Caribbean (Cooley and Kohls, 1944). In the United States, the reported distribution is confined to the southern tip of Texas (Bishopp and Trembley, 1945) (figure 3).

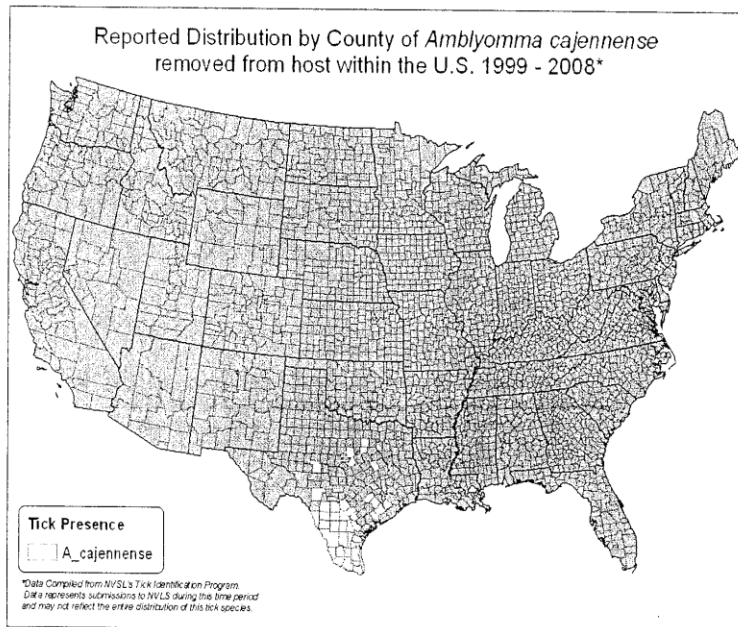


Figure 3

A three-host tick, *A. cajennense* (Fabricius) feeds on equids, but it may infest other mammals such as bovids, cervids, wild and domestic canids, birds, and even humans (Dias and Martins, 1939; Lemos et al., 1997; Rojas et al., 1999; Horta et al., 2004; Guedes et al., 2005). The active immature stages of this tick are indiscriminate in host choice; livestock and a large variety of avian and mammalian wildlife serve as hosts. Although *A. cajennense* is found mainly on horses, Smith (1974) suggested that determining the role of secondary hosts of this tick, maintenance of its biological cycle in the wildlife populations, and the possibility of the dispersion of immature stages by birds was vital for the establishment of appropriate control programs.

A. cajennense is a species that can have economic consequences and is generally quite abundant and active throughout the year. People and livestock can be severely irritated by clusters of *A. cajennense* larvae in wooded and high-grass areas. Most adult ticks attach on the lower body surface, especially between the legs; some feed elsewhere on the body (Evans et al., 2000; Oliveira et al., 2000).

A higher number of males than females were observed on hosts with an increasing ratio of males to females from August and onward. *A. cajennense* males produce pheromones 5 to 6 days after feeding that attract nymphs, females, and males of the same species. Laboratory studies have indicated that *A. cajennense* males can survive for more than 80 days on hosts. Females typically complete their feeding within 2 weeks (Pinter et al., 2002). In some months, certain stages of *A. cajennense* were not present on horses, suggesting that a diapause may occur during some phases of the *A. cajennense* life cycle (Oliveira et al., 2003). Labruna and others (2003) observed 1-year generation times (egg to adult) in Brazil that are primarily controlled by larval behavioral diapauses, as indicated by larval hatching in the spring/summer months. Oliver (1989) suggested that the behavioral diapauses observed in tick populations are adaptive behaviors used to survive unfavorable conditions in the environment (i.e., dry, cold temperatures). Male feeding behavior and larval diapauses should be considered when developing seasonally targeted control programs for tick-borne diseases for equids. Adult cayenne ticks, collected

from EP-positive horses during an outbreak of equine piroplasmiasis on a ranch in south Texas, were allowed to re-attach and feed on a naïve horse, resulting in successful transmission of *Babesia equi* (Scoles, pers. comm., 2010).

Experimental vectors

American dog tick (Dermacentor variabilis)

D. variabilis (Say), the American dog tick, is widely distributed in the United States east of a line drawn from eastern Montana to western South Texas. The tick species is also found in California, west of the Cascade and the Sierra Nevada Mountain ranges and in Canada, east of Saskatchewan (figure 4). This species is most abundant in the eastern United States from Massachusetts south to Florida but is also common in central areas of the United States, including Iowa and Minnesota (Bishopp and Trembley, 1945; Matheson, 1950). The tick also occurs in certain areas of Canada, Mexico, and the U.S. Pacific Northwest (McNemee et al., 2003).

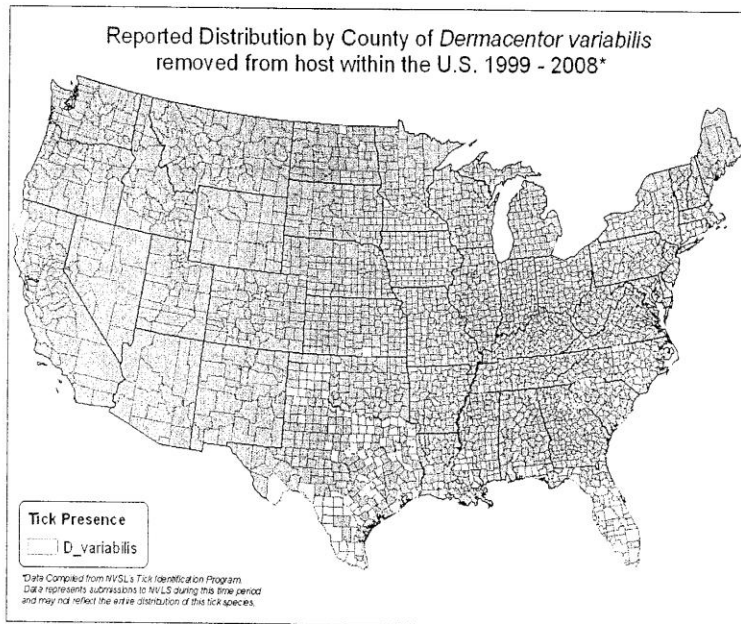


Figure 4

D. variabilis is one of the most frequently encountered ticks by livestock, equids, and humans. *D. variabilis* is a three-host tick with larval and nymphal stages found on a variety of hosts, including mice, birds, chipmunks, voles, squirrels, rats, opossums, and raccoons. Adult ticks are usually found on more medium- to large-sized mammals such as horses, cattle, white-tailed deer, opossums, raccoons, dogs, and humans. Immature ticks prefer rodents, so the abundance of the hosts can be an influencing factor on its distribution and numbers. Meadow voles (*Microtus* sp.) and white-footed mice (*Peromyscus* sp.) are common hosts for immature *D. variabilis*, although they will feed on other species of small mammals. Arthur (1961) indicated that immature stages were found on pine mice, jumping mice, meadow mice, white-footed mice, cotton rats, Norway rats, rabbits, squirrels, and house mice. Adult ticks were found on canids, horses, raccoons, and opossum in Ohio. Immature ticks are typically distributed on the host

around the head region, neck, and shoulders as immatures, while the adults attach around the neck, dewlap, brisket, axillae, groin, genitalia, and abdomen; some feed elsewhere on the body and around the head region (Conlon and Rockett, 1982).

Environmental conditions and terrain can affect tick survival and distribution. High humidity and temperature seem to be important factors for tick survival, as does the abundance of grassy or brush-covered surroundings.

Adult *D. variabilis* winter in the soil and are most active from around mid-April to early September. Larvae are active from about March through July and nymphs are usually found from June to early September (Goddard 1996). In northern areas, such as Massachusetts and Nova Scotia, adults appear from April to August with a peak in May and June (Campbell and MacKay, 1979; Campbell, 1979; McEnroe, 1979a). In central latitudes of the United States such as Virginia, adults are active from April to September/October with peaks in May and July (Sonenshine and Stout, 1971; Carroll and Nichols, 1986). In Ohio, adult activity occurs between April and September with a peak in May and June and a second smaller peak in August and September (Conlon and Rockett, 1982). In Georgia, adult ticks are active from late March to August with peaks from early May to late June (Newhouse, 1983). In Florida, adult *D. variabilis* activity occurs from April to July (McEnroe, 1979b). A study done in Lexington, Kentucky, found the duration of *D. variabilis*' spring activity was related to its overwintering success. This study also concluded that overwintering adult *D. variabilis* ticks remained active throughout the entire season (Burg, 2001). Lastly, the seasonal activity distribution for *D. variabilis* can be bimodal, with peaks of activity occurring twice in a year. In an Ohio study, bimodal peaks occurred in mid-spring and late summer, with a large peak in May and a smaller one in early September. Many of the larvae overwinter, become active in the spring, enter nymphal activity, and molt to adults in late summer with a resulting second peak of adults in early September. Atwood and Sonenshine (1967) found that the questing behavior of *D. variabilis* is positively correlated with the amount of solar radiation received at ground level.

The life cycle of *D. variabilis* requires at least 54 days to complete, but can take up to 2 years depending on host availability, host location, and temperature. In the southern States, the life cycle can be completed in 1 year, with 2 years typical in the northern States (Burg, 2001). The American dog tick occurs primarily in wooded, shrubby, and long-grass areas. However, it is possible for residential areas to support populations of this tick. Shrubs, weeds, tall grass, clutter, and debris attract rodents that are hosts for immature ticks. It has been suggested that adult ticks crawl to the edge of the roads and trails in an attempt to find a host. Some have hypothesized that the scents left by animals following trails attracts these ticks, to the trails (McNemee et al., 2003).

Winter tick (Dermacentor albipictus)

D. albipictus, the winter tick, is widely distributed in the northern and western United States, and in Canada and Mexico. The species is abundant in Maine, Washington, and Oregon. In addition, the dark form of this tick, known previously as *D. nigrolineatus*, can be found in arid parts of Texas, New Mexico, and northern Mexico (figure 5). This tick also is found occasionally on horses and cattle in the southern U.S. States. *D. albipictus* feeds on large mammals like deer, cattle, and horses. This tick species attaches to the host as a larva and remains attached throughout its life (Bishop and Trembley, 1945).

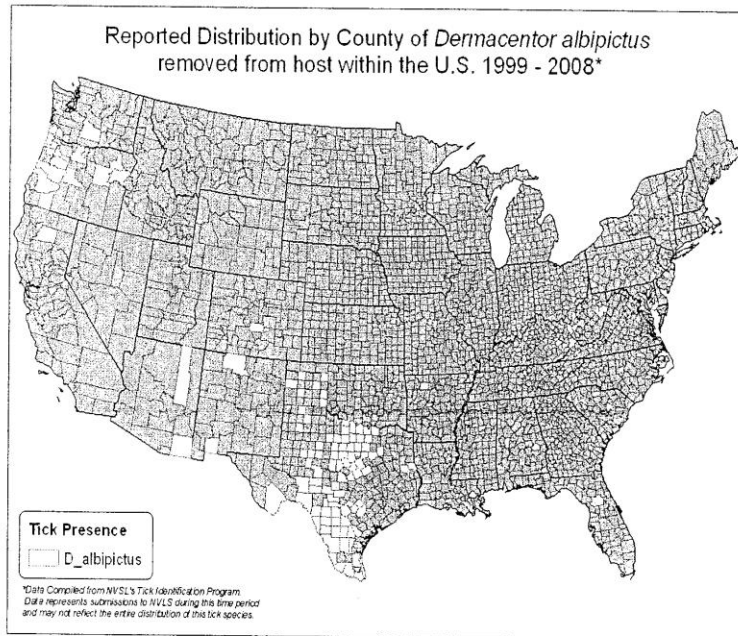


Figure 5

D. albipictus appears to prefer larger hosts such as horses, elk, and moose, but is also abundant on cattle and deer (Bishopp and Trembley, 1945; Gregson, 1956, 1973; Addison and McLaughlin, 1988). Moose and elk can die as a result of heavy infestations of this tick, particularly when the animals' food is in short supply during late winter and early spring. This tick prefers to attach on the dewlap, brisket, abdomen, groin area, and head region, but may be found all over the body in cases of heavy infestation.

The seasonal occurrence of *D. albipictus* on hosts varies with latitude and altitude. During summer, *D. albipictus* exist only as unhatched eggs in the soil. Larvae of this species quest on vegetation in September and October and are quite resistant to cold temperatures. Adults appear by March–April and lay eggs in late June. *D. albipictus* remains on its host from 1 to 2 months, so it can be easily transported to other regions or countries. Development times are sensitive to decreasing photoperiod (i.e., decreasing day length) and freezing soil temperatures. *D. albipictus* is abundant in forested, upland, and mountain habitats, and all stages can occur on large mammals between fall and spring (Wright, 1971; Samuel and Barker, 1979; McPherson et al., 2000).

Ecological implications

For many tick-borne diseases, the presence of the tick alone does not present a sufficient risk for infection. A certain number of host relationships and seasonal dynamics of ticks are required to maintain a robust enzootic cycle. There must be established relationships between environmental conditions and tick-host associations, as both vertebrate host populations and tick questing behavior are influenced by abiotic factors (distributional limits, abundance, and seasonal dynamics) (Sonenshine and Clifford, 1973).

Distribution

A tick's geographical distribution, life history, hosts, and its ability to transmit diseases are determined by intrinsic and extrinsic factors. Intrinsic factors include biochemical and physiological aspects of the tick that determine its reaction to its environment (e.g., the number of offspring produced per reproductive cycle). Extrinsic factors include abiotic and biotic components of the environment that influence the biology of the tick. Climate—temperature, wind, precipitation, humidity, and other meteorological elements—is a main abiotic factor, as it influences horizontal and vertical relationships, life cycle, seasonal activities, population dynamics, and behavior. In addition to climatic factors, the spatial structure of the landscape and its connectivity and vegetation types also have an effect on tick populations and their distributions (Estrada-Pena et al., 2008). The most important biotic factor for a tick is a suitable host or a host that provides a blood source for development and reproduction.

In general, most tick species are less widely distributed than their principal hosts on local and continental scales, with climate appearing to limit a tick's range (Cumming, 1999). Ticks are intermittent parasites, spending part of their lives off their hosts in habitats where they are influenced by abiotic factors. Temperature and rainfall are the main factors affecting the ecology and population dynamics of tick species, and these operate at critical levels on selection of tick populations (Estrada-Pena et al., 2009). For example, the distribution of *Ixodes ricinus* in Britain was associated with several environmental factors, such as substrata composed of less permeable soil types and less permeable superficial/bedrock geology, which would support moist microhabitats. Their distribution was also associated with calcareous/neutral grassland and heathland habitats, particularly those grazed by livestock (Medlock et al., 2008). McEnroe (1977) reported that there were relative differences in the distribution of the American dog tick from region to region, likely due to moisture and temperature differences. Campbell (1979) reported shifts in tick distribution and abundance related to vegetative types and attributed environmental determinants to tick survival.

A tick's distribution or geographic range can be influenced by the physical, immunological, and behavior characteristics of its hosts. Many tick-borne diseases exist in an enzootic state or at a low, stable rate of infection; however, this cycle may change with the advent of a different and opportunistic tick species with a wide variety of acceptable hosts. In contrast, if the host range is narrowed, then transmission may become more focused, resulting in high infection rates in the host (competent reservoir host) and tick vector.

Host specificity may be defined as some form of restriction to a particular class, order, or genus of vertebrate. All ticks do not feed equally on all vertebrate hosts and show some degree of host specificity. As immatures, American dog ticks show a certain amount of host specificity, preferring rodent hosts. For example, in Virginia over 90 percent of the immature American dog ticks were collected from meadow voles and white-footed mice in old field habitats (Sonenshine, 1991). Host specificity may extend from one end of the spectrum (high degree of specificity) to the other (low degree of specificity). The cattle tick, *Boophilus microplus*, preferentially feeds on ruminants, especially cattle, but may also be found on horses when there are horses and cattle in the same pasture (Labruna et al., 2003).

In addition to host preferences, a tick's distribution may be influenced by its host's seasonal behavior. For example, the one-host tick *Dermacentor albipictus* is found on certain wild animals, particularly moose and elk. All life stages of *D. albipictus* occur on these large mammals between fall and spring. *D. albipictus* larvae increase their questing activity in September, concurrent with the period of moose ruts in mid-September to early October (Wright, 1971). During the rut, moose increase their geographical range in search of mates and, with the ticks' increased larval activity during this same period, the contact rate between ticks and hosts increases, as does the ticks' distribution (Bishopp and Trembley, 1945; Gregson, 1956). Moreover, male moose travel farther than females during the rut, which may explain why males average twice as many ticks as females and may have greater influence on the expansion of the ticks' range (Kollars et al., 1997; Cortinas and Kitron, 2006).

Seasonal activity

Many tick species seek hosts during a specific time of year, making transmission of tick-borne diseases seasonal. Understanding the dynamics of these seasonal changes among certain tick populations can help determine the periods when vertebrate hosts are most likely at risk.

Ticks begin to quest for a host after emerging from a diapause. Questing ticks climb vegetation and wait for a host or actively seek a host by walking on the ground. Questing ticks are highly responsive to host CO₂ and body heat. Questing periods vary among each tick's geographic range and by life stage. The seasonal activity of a tick population is controlled by its response to a changing environment, such as photoperiods and ambient temperature. Questing activities can last days or even weeks, depending on the tick's body water loss tolerance. Ticks will return to a moist micro-environment when water loss reaches a certain threshold, and a certain cycle of dehydration-rehydration is not unusual. If environmental conditions become too unfavorable, ticks will enter a diapause rather than continue to seek hosts until the environmental conditions improve (Belozerov, 1982).

The initiation of diapause and the increased seasonal activity of ticks are influenced by a variety of climatic factors. For example, the eggs of the winter tick remain unhatched in the soil during summer and hatch as the temperature and photoperiod decrease in the fall. *Dermacentor albipictus* larvae climb vegetation in autumn and form clumps of more than 100 individuals that attach to passing ungulate hosts (Wright, 1971). The larvae are capable of entering a resting phase or diapause until favorable conditions are present. Larval *D. albipictus* hatch in mid-August to early September in Canada's Elk Island National Park and begin questing in mid-September in response to climatic factors such as photoperiod and above freezing temperatures (Wright, 1971). During the high ungulate host movement activity in the fall, the larvae form clumps around 50 to 190 cm above ground, which coincides with the torso heights of some moose, elk, and deer (McPherson et al., 2000). Larvae rapidly feed and molt to the nymphal stage from 10 to 22 days after attachment. The nymphal stage lasts about 3 months (until January) and thus the nymphal stage enters a diapause on the host during this time, which is likely an adaptation by the ticks to survive colder temperatures. Adults appear on the host from January through March and April (Addison and McLaughlin, 1988). Additionally, Labruna and others (2003) observed larval behavioral diapauses in *A. cajennense* in Brazil, as indicated by larval hatching in the spring and summer as temperatures change. The cayenne tick is quite tolerant of desiccation and can quest for long periods of time in open grazed environment (Needham and Teel, 1991), but their questing activity can vary diurnally and seasonally with climate (Belozerov, 1982).

Diapause is significant not only for survival of the tick vector, but also because it influences the epidemiology of any associated pathogens. Norval and others (1991) indicated that the absence of *Theileria parva* infections in southern Africa may be due to the diapauses in *Rhipicephalus appendiculatus*. *Rhipicephalus appendiculatus* is not continuous throughout the year in southern Africa, with diapauses confined to the unfed adult. Many adults who emerge after July will not quest until November. In contrast, the same tick species in equatorial Africa does not have a diapause. All stages appear throughout the year (Madder et al., 1999). Photoperiods and temperature changes are likely factors that influence diapause activity in ticks (Belozerov, 1982; Fourie et al., 2001).

Abundance

In addition to climatic and landscape changes, changes in biotic factors such as host availability, host abundance, or quality of the blood meal have been proposed as possible determinants for changes in tick abundance (Ogden et al., 2007). Therefore, the population dynamics of ticks are influenced by the seasonality of the host dynamics. Immature American dog ticks can be quite abundant where large numbers of meadow voles exist or in oak/hickory and oak/hickory/pine forests in the southern United States. This increase in the abundance of American dog tick larvae and nymphs in relation to the abundance of the meadow vole in a region can increase the potential for infection of this host (Atwood and Sonenshine, 1967; Sonenshine, 1973). Additionally, tick abundance has been positively correlated with the abundance of larger mammals such as deer in Scotland and the United States (Wilson et al.,

1985; Gilbert, 2010). An increase in the number of individuals within a tick population as an effect of the abundant hosts within a region can effectively develop a zoonotic intensification of tick-borne diseases.

Tick abundance is also limited by abiotic factors that influence the ability to actively quest for a host. Questing ticks are influenced by a variety of abiotic factors such as increasing or decreasing day length or fluctuations in temperature. Additionally, temperatures can change the questing behavior in ticks; for example, decreasing temperature associated with increasing altitude will negatively affect the number of questing numbers of nymphs and adults (Randolph, 2004). Therefore, the opportunity to acquire a pathogen from a reservoir host can be positively or negatively impacted by abiotic factors. It has been observed with *I. ricinus* nymphs that drier conditions impacted the questing height, with more nymphal ticks questing lower and thus having increased exposure to rodents. In contrast, in wetter conditions, an increased number of larvae attached to rodents and fewer nymphs. These climatic factors influenced the questing behavior and thus the potential for a pathogen to be transmitted by nymphs or larvae feeding on rodents. The risk of infection to hosts depends on the number of infected questing ticks. In addition, the larger the number of hosts in an area the greater the probability of ticks attaching and progressing through their life cycle and transmitting a pathogen (Kitron and Kazmierczak, 1997; Randolph, 2001).

Summary

Ecological information on tick species within in the United States is vital for developing and implementing a systematic approach to tick control. The assessment of the developmental times of the free-living tick stages on vegetation will be helpful in determining the major tick species' seasonal activities. With the additional analysis of environmental temperature, rainfall, and tick abundance, this information will aid in the prediction of peak abundance of ticks and the timing of appropriate tick control on a seasonal basis. Moreover, data on life cycles, habitat requirements, and factors influencing distributions and abundance of EP vector ticks would be helpful in the development of predictive models (Petney et al., 1987). This critical information has already been used as a parameter in population models (Sutherst and Maywald, 1985; Floyd et al., 1987) spatial models (Lessard et al., 1990), and climate-driven computerized systems (Sutherst and Maywald, 1985). Through the integration of field, laboratory, and modeling studies, we can gather new insights into the mechanisms of evolutionary ecology regarding the diverse spectrum of tick-borne pathogens, tick species, and their vertebrate hosts.

References—Distribution, Biology, and Ecology of U.S. EP Tick Vectors

- Addison EM, McLaughlin RF, 1988. The growth and development of winter tick, *Dermacentor albipictus*, on moose, *Alces alces*. *J Parasitol* 74:670–678.
- Arthur DR, 1961. Ticks and Disease. New York: Pergamon Press.
- Atwood EL, Sonenshine DE, 1967. Activity of the American dog tick, *Dermacentor variabilis* (Acarina: Ixodidae) in relation to solar energy changes. *Ann Entomol Soc Am* 60(2):354–362.
- Barker SC, Murrell A, 2004. Systematics and evolution of ticks with a list of valid genus and species names. *Parasitol.* 129:15–36.
- Battsetseg B, Lucero S, Xuan X, Claveria FG, Inoue N, Alhassan A, Kanno T, Igarashi I, Nagasawa H, Mikami T, Fujisaki K, 2002. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. *Vet Parasitol* 107: 351–357.
- Belozero VN, 1982. Diapause and biological rhythms in ticks. In *The Physiology of Ticks*, ed. Obenchain FD, Galun R. Pergamon Press, Oxford, England, 469–500.
- Bishopp FC, Trembley HL, 1945. Distribution and hosts of certain North American ticks. *J Parasitol* 31:1–54.
- Borges LM, Oliveira PR, Lisboa CL, Ribeiro MF, 2002. Horse resistance to natural infestations of *Anocentor nitens* and *Amblyomma cajennense* (Acari: Ixodidae). *Vet Parasitol* 104(3):265–73.
- Bryant JE, Anderson JB, Willers KH, 1969. Control of equine piroplasmiasis in Florida. *J Am Vet Med Assoc* 154:1034–1036.
- Burg JG, 2001. Seasonal activity and spatial distribution of host-seeking adults of the tick *Dermacentor variabilis*. *Med Vet Entomol* 15: 13–421.

- Campbell A., 1979. Ecology of the American dog tick, *Dermacentor variabilis* in southwestern Nova Scotia. In Recent Advances in Acarology, ed. Rodriguez JG. 2:135–143.
- Campbell A, MacKay PR, 1979. Distribution of the American dog tick, *Dermacentor variabilis* (Say), and its small-mammal hosts in relation to vegetation types in a study area in Nova Scotia. *Can J Zool* 57:1950–1959.
- Carroll JF, Nichols JD, 1986. Parasitization of meadow voles, *Microtus pennsylvanicus* (Ord), by American dog ticks, *Dermacentor variabilis* (Say), and adult tick movement during high host density. *J Entomol Sci* 21:102–113.
- Conlon JM, Rockett CL, 1982. Ecological investigations of the American dog tick, *Dermacentor variabilis* (Say), in northwest Ohio (Acari: Ixodidae). *Internat J Acarol* 8:125–131.
- Cooley RA, Kohls, GM, 1944. The Argasidae of North America, Central America, and Cuba. *Am Midl Nat Monogr* 1:1–152.
- Cortinas MR, Kitron U, 2006. County-level surveillance of white-tailed deer infestation by *Ixodes scapularis* and *Dermacentor albipictus* (Acari: Ixodidae) along the Illinois River. *J Med Entomol* 43:810–819.
- Cumming GS, 1999. Host distributions do not limit the species ranges of most African ticks (Acari: Ixodida). *Bull Entomol Res* 89:303–327.
- Dennis DT, Nekomoto TS, Victor JC, Paul WS, Piesman J. 1998. Reported distribution of *Ixodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae) in the United States. *J Med Entomol* 35:629–638.
- DeWaal DT, 1992. Equine piroplasmiasis: a review. *Br Vet J* 148:6–14.
- Dias E, Martins AV, 1939. Spotted fever in Brazil. *Am J Trop Med* 1–19(2):103–108
- Estrada-Pena A, 2006. Prediction of habitat suitability for ticks. *Ann New York Acad Sci* 1078:275–284.
- Estrada-Pena A, 2008. Climate, niche, ticks, and models: what they are and how we should interpret them. *Parasitol Res* 103(1):587–595.
- Estrada-Pena A, Naranjo V, Acevedo-Whitehouse K, Mangold AJ, Kocan KM, de la Fuente J, 2009. Phylogeographic analysis reveals association of tick-borne pathogen, *Anaplasma marginale*, MSP1a sequences with ecological traits affecting tick vector performance. *BMC Biol* 7:57.
- Evans DE, Martins JR, Guglielmo AA, 2000. A review of the ticks (Acari, Ixodida) of Brazil, their hosts and geographic distribution—I. The State of Rio Grande do Sul. *S Brazil Mem Inst Oswaldo Cruz* 95(4):453–470.
- Floyd RB, Maywald GF, Sutherst RW, 1987. Ecological models. 2. A population model of *Rhipicephalus appendiculatus*. In Sutherst, RW, ed. Ticks and Tick-Borne Diseases: Proceedings of an International Workshop on the Ecology of Ticks and the Epidemiology of Tick-Borne Diseases. Australian Centre for International Agricultural Research Proceedings 17:72–75.
- Fourie LJ, Belozero VN, Needham GR, 2001. *Ixodes rubicundus* nymphs are short-day diapause-induced ticks with thermolabile sensitivity and desiccation resistance. *Med Vet Entomol* 15:335–341.
- Friedhoff KT, Tenter AM, Muller I, 1990. Haemoparasites of equines: impact on international trade of horses. *Revue Scientifique et Tech OIE* 9:1187–1194.
- Gilbert L, 2010. Altitudinal patterns of tick and host abundance: a potential role for climate change in regulating tick-borne diseases? *Oecologia* 162:217–225.
- Goddard J, 1996. Physician's Guide to Arthropods of Medical Importance. CRC Press, Jackson, MS. 287–302.
- Gregson JD, 1956. The Ixodoidea of Canada. Entomology Division, Science Service, Canada Department of Agriculture, Publication 930:92.
- Gregson JD, 1973. Tick paralysis: an appraisal of natural and experimental data. *Can Dept Agric Monograph* 9, Ottawa.
- Guedes E, Leite RC, Prata MCA, Pacheco RC, Walker DH, Labruna MB, 2005. Detection of *Rickettsia rickettsii* in the tick *Amblyomma cajennense* in a new Brazilian spotted fever—endemic area in the state of Minas Gerais. *Mem Inst Oswaldo Cruz* 100:841–848.
- Guimaraes AM, Lima JD, Ribeiro MFB, 1998. Sprogony and experimental transmission of *Babesia equi* by *Boophilus microplus*. *Parasitol Res* 84:323–327.
- Heuchert CMS, Giulli V, Athaide DF, Bose R, Friedhoff KT, 1999. Seroepidemiologic studies on *Babesia equi* and *Babesia caballi* infections in Brazil. *Vet Parasitol* 85:1–11.
- Holbrook AA, Johnson AJ, Madden BS, 1968. Equine piroplasmiasis: Intraerythrocytic development of *Babesia caballi* (Nuttall) and *Babesia equi* (Laveran). *Am J Vet Res* 29:297–303.

- Horak IG, Camicas JL, Keirans JE, 2002. The Argasidae, Ixodidae, and Nuttalliellidae (Acari: Ixodida): a world list of valid tick names. *Exp Appl Acarol* 28:(1-4):27–54.
- Horta MC, Labruna MB, Sangioni LA, Vianna MCB, Gennari MS, Galvão MAM, Mafra C, Vidotto O, Schumaker TT, Walker DH, 2004. Prevalence of antibodies to spotted fever group rickettsiae in humans and domestic animals in a Brazilian spotted fever endemic area in the state of São Paulo, Brazil: serological evidence for infection by *Rickettsia rickettsii* and another spotted fever group rickettsia. *Am J Trop Med Hyg* 71:93–97.
- Keirans JE, Durden LA, 2001. Invasion: exotic ticks (Acari: Argasidae, Ixodidae) imported into the United States. A review and new records. *J Med Entomol* 38(6):850–861.
- Kitron U, Kazmierczak JJ, 1997. Spatial analysis of the distribution of Lyme disease in Wisconsin. *Am J Epidemiol* 145(6):558–566.
- Klompen JS, Black WC IV, Keirans JE, Oliver JH Jr, 1996. Evolution of ticks. *Ann Rev Entomol* 41:141–161.
- Knowles DP Jr, Kappmeyer LS, Stiller D, Hennager SG, Perryman LE, 1992. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*.
- Knowles RC, Mathis RM, Bryant JE, Willers KH, 1966. Equine piroplasmiasis. *J Am Vet Med Assoc* 148(4):407–410.
- Kollars TM Jr, Durden LA, Masters EJ, Oliver JH Jr, 1997. Some factors affecting infestation of white-tailed deer by blacklegged ticks and winter ticks (Acari: Ixodidae) in southeastern Missouri. *J Med Entomol* 34:372–375.
- Labruna MB, Amaku M, Metzner JA, Pinter A, Ferreira F, 2003. Larval behavioral diapause regulates life cycle of *Amblyomma cajennense* (Acari: Ixodidae) in Southeast Brazil. *J Med Entomol* 40(2):170–178.
- Labruna MB, Kasai N, Ferreira F, Faccini JL, Gennari SM, 2002. Seasonal dynamics of ticks (Acari: Ixodidae) on horses in the state of São Paulo, Brazil. *Vet Parasitol* 105(1):65–77.
- Labruna MB, Terassini FA, Camargo LM, 2009. Notes on populational dynamics of *Amblyomma* ticks (Acari: Ixodidae) in Brazil. *J Parasitol* 3:1.
- Lemos ER, Machado RD, Coura JR, Guimarães MA, Freire NM, Amorim M, Gazeta GS, 1997. Epidemiological aspects of the Brazilian spotted fever: seasonal activity of ticks collected in an endemic area in São Paulo, Brazil. *Rev Soc Bras Med Trop* 30:181–185.
- Matheson R, 1950. *Medical Entomology*, 2nd ed. Comstock Publishing Company, Inc., Ithaca, NY.
- McEnroe WD, 1977. Human babesiosis. *Science* 195:506–507.
- McEnroe WD, 1979a. *Dermacentor variabilis* (Say) in eastern Massachusetts. Recent Advances in Acarology, ed. Rodriguez JG. 2:145–153.
- McEnroe WD, 1979b. The effect of the temperature regime on *Dermacentor variabilis* (Say) populations in eastern North America. *Acarologia* 20:58–67.
- McNemee RB, Sames WJ, Maloney FA Jr, 2003. Occurrence of *Dermacentor variabilis* (Acari: Ixodidae) around a porcupine (Rodentia: Erthethizontidae) carcass at Camp Ripley, Minnesota. *J Med Entomol* 40:108–111.
- McPherson M, Shostak AW, Samuel WM, 2000. Climbing simulated vegetation to heights of ungulate hosts by larvae of *Dermacentor albipictus* (Acari: Ixodidae). *J Med Entomol* 37:114–120.
- Medlock JM, Pietzsch ME, Rice NV, Jones L, Kerrod E, Avenell D, Los S, Ratcliffe N, Leach S, Butt T, 2008. Investigation of ecological and environmental determinants for the presence of questing *Ixodes ricinus* (Acari: Ixodidae) on Gower, South Wales. *J Med Entomol* 45:314–325.
- Murrell A, Barker SC, 2003. Synonymy of *Boophilus* Curtice, 1891 with *Rhipicephalus* Koch, 1844 (Acari: Ixodidae). *Syst Parasitol* 56:169–172.
- Needham GR, Teel PD, 1991. Off-host physiological ecology of ixodid ticks. *Ann Rev Entomol* 36:659–681.
- Neitz WO, 1956. Classification, transmission, and biology of piroplasms of domestic animals. *Ann NY Acad Sci* 64:56–111.
- Newhouse VF, 1983. Variations in population density, movement, and rickettsial infection rates in a local population of *Dermacentor variabilis* (Acarina: Ixodidae) ticks in the piedmont of Georgia. *Envir Entomol* 12:1737–1746.
- Norval RA, Lawrence JA, Young AS, Perry BD, Dolan TT, Scott J, 1991. Theileria parva: influence of vector, parasite and host relationships on the epidemiology of theileriosis in southern Africa. *Parasitol* 102(3):347–356.

-
- Ogden NH, Bigras-Poulin M, O'Callaghan CJ, Barker IK, Kurtenbach K, Lindsay LR, Charron DF, 2007. Vector seasonality, host infection dynamics and fitness of pathogens transmitted by the tick *Ixodes scapularis*. *Parasitol* 134(2):209–227.
- Oliveira PR, Borges LM, Lopes CM, Leite RC, 2000. Population dynamics of the free-living stages of *Amblyomma cajennense* (Fabricius, 1787) (Acari: ixodidae) on pastures of Pedro Leopoldo, Minas Gerais State, Brazil. *Vet Parasitol* 92(4):295–301.
- Oliveira PR, Borges LM, Leite RC, Freitas CM, 2003. Seasonal dynamics of the Cayenne tick, *Amblyomma cajennense* on horses in Brazil. *Med Vet Entomol* 17(4):412–416.
- Oliver JH, 1989. Biology and systematics of ticks (Acari: Ixodida). *Ann Rev Ecol Syst* 20:397–430.
- Petney TN, Horak IG, Rechav Y, 1987. The ecology of the African vectors of heartwater, with particular reference to *Amblyomma hebraeum* and *Amblyomma variegatum*. Onderstepoort *J Vet Res* 54(3):381–395.
- Pinter A, Labruna MB, Faccini JL., 2002. The sex ratio of *Amblyomma cajennense* (Acari: Ixodidae) with notes on the male feeding period in the laboratory. *Vet Parasitol* 19;105(1):79–88.
- Randolph SE, 2001. The shifting landscape of tick-borne zoonoses; tick-borne encephalitis and Lyme borreliosis in Europe. *Phil Trans R Soc Lond B Biol Sci* 356(1411):1045–1056.
- Randolph SE, 2004. Evidence that climate change has caused 'emergency' of tick-borne diseases in Europe? *Int J Med Microbiol* 37:5–15.
- Roby TO, Anthony DW, 1963. Transmission of equine piroplasmiasis by *Dermacentor nitens* Neumann. *J Am Vet Med Assoc* 142:768–769.
- Rojas R, Marini MA, Zanatta Coutinho AT, 1999. Wild birds as hosts of *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae). *Mem Inst Oswaldo Cruz* 94(3):315–322.
- Rowe, R., pers. comm., 2010.
- Samuel WM, Barker MJ, 1979. The winter tick, *Dermacentor albipictus* (Packard, 1869) on moose, *Alces alces* (L.) of central Alberta. *Proc N Am Moose Conf Wkshp* 15:303–348.
- Smith MW, 1974. Some aspects of the ecology and lifecycle of *Amblyomma cajennense* (Fabricius, 1787) in Trinidad and their influence on tick control measures. *Ann Trop Med Parasitol* 69:121–129.
- Sonenshine DE, 1991. *Biology of Ticks*. v 1- 2. Oxford University Press, New York.
- Sonenshine DE, Clifford CM, 1973. Contrasting incidence of Rocky Mountain spotted fever in ticks infesting wild birds in eastern U.S. Piedmont and coastal areas, with notes on the ecology of these ticks. *J Med Entomol* 10:497–502.
- Stiller D, Coan ME, 1995. Recent developments in elucidating tick vector relationships for anaplasmosis and equine piroplasmiasis. *Vet Parasitol* 57:97–108.
- Stiller D, Goff WL, Johnson LW, Knowles DP, 2002. *Dermacentor variabilis* and *Boophilus microplus* (Acari: Ixodidae): experimental vectors of *Babesia equi* to equids. *J Med Entomol* 39:667–670.
- Strickland RK, Gerrish RR, 1964. Distribution of the tropical horse tick in the United States, with notes on associated cases of equine piroplasmiasis. *J Am Vet Med Assoc* 144:875–878.
- Sutherst RW, Maywald GF, 1985. A computerized system for matching climates in ecology. *Ag Ecosys Envir* 13:381–299.
- Taylor WM, Bryant JE, Anderson JB, Willers KH, 1969. Equine piroplasmiasis in the United States—a review. *J Am Vet Med Assoc* 155:915–919.
- Thompson PH, 1969. Ticks as vectors of equine piroplasmiasis. *J Am Vet Med Assoc* 155(2):454–457.
- Ueti MW, Palmer GH, Kappmeyer LS, Statfield M, Scoles GA, Knowles DP, 2005. Ability of the vector tick *Boophilus microplus* to acquire and transmit *Babesia equi* following feeding on chronically infected horses with low-level parasitemia. *Clin Microbiol* 43(8):3755–3759.
- Ueti MW, Palmer GH, Scoles GA, Kappmeyer LS, Knowles DP, 2008. Persistently infected horses are reservoirs for intrastadial tick-borne transmission of the apicomplexan parasite *Babesia equi*. *Infect Immun* 76(8):3525–3529.
- Utech KBW, Sutherst RW, Dallwitz MJ, Wharton RH, Maywald GF, Sutherland ID, 1983. A model of the survival of larvae of the cattle tick, *Boophilus microplus*, on pasture. *Austr J Agric Res* 34:63–72.
- Wilson ML, Adler GH, Spielman A, 1985. Correlation between abundance of deer and that of the deer tick, *Ixodes dammini* (Acari: Ixodidae). *Ann Entomol Soc Am* 78:172–176.
- Wright JE, 1971. Relationship of seasonal activity of *Dermacentor albipictus* (Acarina; Ixodidae) to photoperiod: a laboratory study. *Ann Entomol Soc Am* 64:1456–1458.

Control of Tick Vectors of Equine Piroplasmiasis

As ectoparasites of equids, ticks are difficult to control because they are small—especially during early developmental stages—which makes them a challenge to find on or off a host, and because they can be attached to a host for varying time periods. An overview of tick species commonly found on companion animals is provided by Dryden and Payne (2004).

In the case of multihost ticks, one or more larval, nymphal, or adult stages may each involve a different host, depending on feeding preferences and host availability. When multihost ticks are not blood feeding, they are normally free living in the environment, further complicating detection and effective control. Since ticks have seasonal patterns of abundance, it is important to select control strategies that are most effective against a specific developmental stage before pathogen transmission might occur. In the case of tick species that seek different hosts, awareness of the common questing practices can help in the development of management practices that minimize host exposure to ticks. For one-host ticks, each life stage may remain on the same host after feeding, and early detection followed by preventive treatment is critical. Varying host preferences among one- and three-host tick vectors make it unlikely that any single control strategy will succeed. Rather, a range of control methods is needed to prevent equine tick exposure and to avoid pathogen transmission.

Examining horses for ticks

USDA guidance on the inspection of horses for ticks recommends a thorough and systematic examination. The following examination procedure, known as “scratching” for ticks, has been recommended (Rowe, personal communication, 2010):

- Beginning at the horse's head, examine the both ears and palpate inside of each ear, examine the false nostrils visually and palpate with the forefinger.
- Move to the forelock and, with thumb opposed to fingers, examine the forelock, continuing down the mane to the withers.
- Examine the submandibular/intermandibular space using flattened hand and fingers, feeling for any unevenness of the skin.
- Examine the axilla of one side, visually and through palpation.
- Examine the posterior fetlock to the coronet of the front foot, visually and through palpation.
- Visually examine the udder/scrotum area on one side.
- Examine the tail and perineum, visually and through palpation.
- Examine the posterior fetlock to the coronet of the back foot, visually and through palpation
- Examine the udder/scrotum of the other side.
- Examine the posterior fetlock to the coronet of the other back foot, visually and through palpation.
- Examine the posterior fetlock to the coronet of the other front foot, visually and through palpation.
- Examine the axilla of the other side, visually and through palpation.
- Perform hand hygiene after examining each animal.

Removing attached ticks

Many of the methods used to remove attached ticks from a host are based on folklore and might cause additional harm to the animal. Some of the common folk methods include applying petroleum jelly, fingernail polish, 70 percent isopropyl alcohol, or a hot kitchen match to the attached tick (Needham, 1985). While these methods are thought to induce a tick to “back out” of an attachment site, they actually stimulate a tick to secrete more saliva, cause regurgitation, or introduce other tick-body secretions or excretions into the wound. These procedures increase the risk of secondary infection around the bite

location. The best method, as described by Needham (1985), is to implement the following procedures in sequence:

1. Using blunt curved forceps, grasp the tick as close to the skin surface as possible and pull upward with a steady even pressure.
2. Avoid squeezing, crushing, or puncturing the tick's body.
3. Do not handle the tick with bare hands.
4. Cleanse the bite site with soap and water.

As a possible alternative to forceps, Stewart and others (1998) compared three commercial tick-extraction tools (Ticked-Off, Tick Nipper, and Pro-Tick Remedy) and found that all three were an improvement over forceps. These tools worked well for removing nymphs and adults by taking advantage of the tick's mouthpart morphology and body size to minimize mistakes that can be made when using forceps. One or more of these tools are available at outdoor and camping retail stores. Another method for removing ticks is spraying the ticks with pyrethrins or pyrethroids containing aerosol repellent, then spraying again within 1 minute. Ticks will fall off after treatment.

To submit the sample for identification, place the tick in a container of alcohol (optimally 70 percent ethanol or 70 percent isopropyl) and send the specimen to a State or Federal (National Veterinary Services Laboratories) laboratory responsible for tick identification.

Preventing tick infestations

Preventing equine tick exposure is complicated by the specific biological requirements of a tick species and whether a tick's life cycle is dependent on one host or multiple hosts. If a tick species utilizes multiple hosts, then host availability, access to suitable questing sites, and seasonal changes in tick abundance play a role in the likelihood of an equid becoming infested with ticks. A study by Labruna and others (2001) identified risk factors related to tick infestation of horses in São Paulo, Brazil. *A. nitens*, *A. cajennense*, and *B. microplus* were the only species feeding on horses, with *A. nitens* being the most abundant of the three species. *A. cajennense* ticks were most abundant in pastures with a mixed overgrowth of grasses and shrubs, and tick abundance on pasture vegetation was correlated with levels of tick infestation on horses. The authors found that mowing pastures once a year was an effective means of avoiding high infestations of *A. cajennense*. *B. microplus* infestations on horses were correlated with the presence of cattle on a shared pasture. The abundance of *A. nitens* ticks did not correlate with any of the risk factors evaluated in this study. It was also observed that *A. cajennense* infestations on horses were higher on farms that sprayed acaricide on horses. Similarly, applying a topical acaricide did not control *A. nitens* infestations; however, the authors could not confirm whether the topical treatments were applied as directed by the manufacturer's label.

A study by Lopes and others (1998) emphasized the important role of secondary hosts in dispersing the three-host tick *A. cajennense* to new areas. In areas where *A. cajennense* was present, one or more stages of this tick species were commonly found attached to equids, small mammals, birds, and domestic animals. The duration of attachment, or drop-off periodicity, varied by host species and a tick's life stage. On average, larvae and nymphs remained attached for 3 to 4 days. Results for the attachment times of adult ticks were inconclusive.

Nonchemical control of ticks

Information on a wide variety of tick control strategies is summarized in the "Tick Management Handbook" (Stafford, 2007) developed by the Connecticut Agricultural Experiment Station. For the purposes of this report, control methods directed primarily at populations of *Ixodes scapularis*, *Dermacentor variabilis*, and *Amblyomma americanum* ticks relative to Lyme disease and other zoonotic diseases will be emphasized. This report recommends viewing an area of possible tick exposure as a "tickscape." Using this approach, the potential of grasses, shrubs, and other vegetation to serve as tick habitat is considered in a landscape management plan. Modifications to desirable tick habitats serve to reduce the abundance of questing ticks and lessen host exposure.

Tickscape management plans should include habitat edges (i.e., ecotones) where a greater diversity of vegetation types is likely to occur. In addition to habitat management, other nonchemical methods of tick control focus on reducing populations or limiting movements of free-ranging wildlife hosts, such as deer. Examples of host-limiting habitat modifications include deer fencing, deer repellents, and substituting less palatable plants in a landscape. Although this approach works well in suburban residential landscapes, it might be impractical in rural areas.

Controlling ticks by using plants that ticks avoid has been tried in South Africa. Moyo and Masika (2009) reviewed various tick control methods used by resource-limited farmers in Eastern Cape Province, South Africa. Farmers there reported that the leaves of *Aloe ferox* (commonly known as Cape Aloe), a medicinal plant of the Asphodelaceae family, has insect and tick repellent properties. In addition, the bark of *Ptaeroxylon obliquum*, a member of the Rutaceae family, has repellent and acaricidal properties. Although no reports could be found on the incorporation of repellent and acaricidal plants in a landscape in the United States, this approach is worthy of consideration and further investigation.

Chemical control of ticks

Chemical control methods are effective in reducing host exposure to ticks, especially when appropriate chemicals are applied at times and locations that will have the greatest impact on the developmental stages of a tick, either on or off a host. To control multihost ticks that spend various amounts of time off a host, area-wide acaricides may be applied to tick habitats, such as woodland edges and grassy patches located near riparian areas. Area-wide acaricide treatments should correspond to times when larval and nymphal tick stages are most active. All chemicals used as insecticides or acaricides must be registered with the U.S. Environmental Protection Agency (EPA) and must be approved for areawide use or for use on a specific host species.

The most common compounds used in areawide control are carbamate insecticides (e.g., Sevin[®] with the carbaryl, 1-naphthyl methylcarbamate, as the active ingredient); natural pyrethrins (derived from seed cases of the *Chrysanthemum cinerariaefolium* plant and consisting of a combination of two stereoisomers, pyrethrin I and pyrethrin II, along with a cyclopropane core); and synthetic pyrethrins (often called pyrethroids). Pyrethrins have been recognized as neurotoxins of insects for nearly a century, following the report by Staudinger and Ružička (1924) of the insecticidal property of this natural compound. Staudinger and Ružička also reported that in low or nonlethal doses, pyrethrins had repellent properties. Pyrethrins are nonpersistent because they break down quickly when exposed to light and oxygen. Although pyrethrins do not persist in the environment, these compounds are toxic to many aquatic organisms, waterfowl, and bees (Aldridge, 1990; Anonymous, 1994). While human toxicity to pyrethrins is considered low, fatal asthma has been reported after the use of an animal shampoo containing pyrethrin (Wagner, 2000).

A recent search of the National Pesticide Information Retrieval System was used to identify which EPA-registered pesticides are approved for the control of ticks on horses (Messenger, pers. comm., 2010). Active chemical ingredients in compounds registered with the EPA for the control of ticks on horses include: natural pyrethrins, pyrethroids (synthetic pyrethrins), coumaphos (an organophosphate insecticide), sulfur (a wettable sulfur powder), and tetrachlorvinphos (an organophosphate in a dust or wettable powder). These active ingredients are included in various formulations and marketed under various brand names. The acaricides on this list are also registered for use on cattle; however, more chemical compounds are registered for use on cattle than on horses. An important acaricide approved for use on cattle is amitraz (a triazapentadiene compound) which cannot be used on horses because it can cause irreversible gut stasis. The most common acaricide formulations for use on horses are natural and synthetic pyrethrins. Often these formulations include piperonyl butoxide as a synergist that inhibits rapid metabolism of the active compound by arthropods, thus prolonging the neurotoxic effects. Other synergists that may be added are: chloropyrifos, dichlorvos, or thiazolyn (Graf et al., 2004). Pyrethroid formulations frequently used for tick control on horses are: cypermethrin, deltamethrin, alphasmethrin, tetramethrin, and prallethrin.

There are no systemic acaricides registered for internal use on cattle or horses. The high cost and the long development time needed to identify and synthesize potential acaricides, along with efficacy, safety, and environmental studies, have hindered the development of new products that control ticks on cattle or horses (Graf et al., 2005). Limiting factors in the development of new acaricidal products include improving product efficacy, reducing chemical resistance, and ensuring the safety of horses, humans, and the environment. When spraying a horse with an acaricide, all skin surfaces should be wetted, including the undercarriage. Acaricide should be wiped onto the surfaces of the pinna and false nostril. Dipping is the optimal method for applying acaricides, as this method ensures that all skin surfaces are wetted; however, this method is seldom used on horses.

A laboratory study by Drummond (1988) compared 17 formulations of 15 acaricides on 7 species of engorged female ticks. Effectiveness was measured as a reduction in the number of eggs produced per engorged female after exposure to a candidate acaricide. The most effective acaricides were chlorfenvinphos, lindane, chlorpyrifos, coumaphos, diazinon, permethrin, phosmet, amitraz, dioxathion, arsenic trioxide, malathion, tetrachlorvinphos, carbaryl, toxaphen, and ronnel. Tick species tested included *A. cajennense*, *A. nitens*, *B. annulatus*, and *B. microplus*. All compounds tested demonstrated toxicity to ticks; however, except for permethrin and coumaphos, these compounds do not meet EPA requirements for low human health risk, rapid environmental degradation, and low ecological toxicity to nontarget organisms (EPA, docket number: EPA-HQ-OPP-2008-0023; EPA 738-R-06-017, 2007). Studies by Jongejan and Uilenberg (1994) demonstrated that levels of tick infestation are usually decreased through the alternate use of chemical acaricides on animals and in the environment, while considering the seasonal dynamics of ticks. Nevertheless, these authors found the use of acaricides to be unsatisfactory due to problems of acaricide resistance in ticks, animal product contamination, and environmental residues.

Antiparasitic drugs such as ivermectin are approved for internal use in horses for controlling gastrointestinal parasites. The insecticidal and acaricidal properties of this macrocyclic lactone compound (22,23-dihydroavermectin B 1a + 22,23-dihydroavermectin B 1b) have been reported to be effective in the control of ticks in cattle and horses. Experimental studies on cattle conducted by Drummond (1985) demonstrated ivermectin's effectiveness against cattle grubs, mites, and eight species of ticks. Subcutaneous injections of ivermectin produced 95- to 100-percent control at lower doses than when this drug was administered by the oral route. Campbell and Benz (1984) reviewed the efficacy and safety of ivermectin in cattle, horses, sheep, swine, and dogs. The antiparasitic efficacy of ivermectin in the control of nematodes, mites, ticks, and parasitic flies was summarized for cattle. However, in horses the authors only reported efficacy studies to parasitic helminths, mostly intestinal nematodes. Efficacy of ivermectin in the control of ticks on horses was not reported.

USDA guidelines (USDA, 2008) for treating cattle, horses, and sheep with acaricides recommend dipping animals in vats containing an approved acaricide and strictly following the concentration amounts recommended on the label. When horses are imported to the United States for special events, APHIS-VS (USDA, 2003) requires all horses to be free of ticks or other external parasites. To ensure that this requirement is met, horses must be sprayed with a solution of dioxathion (p-dioxane-2,3 diyl ethyl phosphorodithioate) [trade name Delnav[®]], an organophosphate acaricide, which is also used to control insects and mites on citrus fruits, deciduous fruits, and nuts. An acceptable alternative to dioxathion is the use of 32.5-percent emulsifiable concentrate of Atroban[®], a broad spectrum pesticide composed of permethrin (0.05 percent). In addition, veterinary examiners inspecting horses imported for special events must check the false nostril and external ear canal (inside pinna) for ticks and then treat these areas with one of the approved acaricides mentioned previously.

Use of vaccines in tick control and disease prevention

Another approach to controlling tick populations is inducing host-acquired immunity against ticks by active immunization with either crude, purified native, or recombinant antigens derived from ticks (Mulenga et al., 2000). Consequently, studies have focused on potent antigens for the development of efficacious vaccines (Willadsen, 2004). Two vaccines are commercially available for cattle: TickGARD Plus, (Rodriguez et al., 1995) and Gavac Plus (Willadsen et al., 1995), developed and tested in Cuba and

Australia, respectively. Both vaccines are constituted of the Bm86 antigen, which is a membrane-bound glycoprotein of 89kDa on the microvilli of tick-gut digestive cells (Rand et al., 1989; Willadsen et al., 1989; Gough and Kemp, 1993). Use of Bm86 antigen vaccine reduces the numbers and weights of engorged ticks, decreases egg laying capacity, and lowers egg viability leading to a decline of the *B. microplus* population (Kemp et al., 1989; Willadsen et al., 1995). An efficacy rate of 99 percent has been reported by the majority of studies testing the Bm86 antigen-based vaccine (Fragoso et al., 1998; de la Fuente et al., 2000a; de Vos et al., 2001; Canales et al., 2009).

The Bm86 antigen was originally identified in the cattle tick *B. microplus* and was successfully used against this same species; however, vaccine efficacy is inconsistent and usually much lower when using this antigen, or homologues of the antigen, in immunizing animals against other tick species, particularly in the most important genera of *Amblyomma*, *Hyalomma*, and *Rhipicephalus*. Vaccination has had variable efficacy, depending on the tick species used when testing vaccine efficacy (de Vos et al., 2001). This observation suggests that tick genetic and/or physiological differences may affect the efficacy of tick vaccines in different geographic regions (Fragoso et al., 1998; García-García et al., 1999, 2000; de la Fuente et al., 1999, 2000a, 2000b; de Vos et al., 2001; de la Fuente and Kocan 2006; Sossai et al., 2005; Canales et al., 2009). Several tick-antigen targets considered to date are from a limited range of functional classes. Such antigens consist of structural proteins, predominantly from salivary glands; hydrolytic enzymes and their inhibitors, mainly those implicated in hemostatic processes; and a group of membrane-associated proteins of indefinite function (Willadsen, 2006). Moreover, the use of an antigen mix to increase vaccine efficacy has been the subject of numerous experimental trials, with results that have been ambiguous and inconclusive (Willadsen, 2008).

Despite having new technologies available to identify antigens capable of eliciting a protective immune response against a tick infestation, the number of well-characterized antigens remains small and constitutes a significant challenge (Hope et al., 2010). Improving the existing Bm86 antigen vaccine is likely to rely on the discovery of other antigens that in combination might offer improved and multispecies efficacy. The choice of target antigens depends on whether an immunizing molecule can protect the host against both tick infestation and pathogen transmission. An ideal vaccine would be capable of reducing tick populations and protecting against infection by tick-borne pathogens and/or affecting tick vectorial capacity (de la Fuente et al., 1998, 2007; Rodríguez Valle et al., 2004). The high cost and technical problems associated with screening a sizeable number of tick-protective antigen candidates along with vaccination trials constitutes a major obstacle to obtaining approval for new vaccines. In recent research by Almazan and others (2010), antitick vaccination was shown to be a cost-effective alternative for the control of tick infestations, reducing the use of chemical acaricides and preventing selection of drug resistant ticks (de la Fuente et al., 2007).

Summary and recommendations

Tick vectors of EP pathogens are difficult to detect on equids and in their environment, and control is a challenge. The best approach is to use the principles of integrated pest management and incorporate multiple strategies aimed at various life stages, multiple hosts, and a variety of landscapes. Limiting tick access to equine hosts by developing landscapes poorly suited to tick-host interaction and use of tick repellents or repelling acaricides are initial approaches to avoiding exposure. Ivermectin shows promise in the systemic control of blood-feeding ticks. Although ivermectin has potential as a method of tick control, the impact of this compound on the epidemiology of EP is uncertain, because an infected tick may still transmit sporozoites to a host before being affected by ivermectin. In addition, ivermectin is not known to have antiparasitic properties against *B. caballi* or *B. equi*. Meanwhile, efforts should be made to obtain formal approval of ivermectin as a systemic acaricide. For long-term prevention and control, it is important to have one or more vaccines directed against blood-feeding ticks and against *B. caballi* and *T. equi* sporozoites, or other stages of the hemoprotozoans that can be used to elicit a protective immune response. More research is needed in the development of effective multispecies tick vaccines that protect horses from both tick vectors and the infectious organisms that cause piroplasmosis. Having preventive tickscapes, effective repellents, external acaricides, systemic acaricides, and specific vaccines will allow for the use of integrated pest management strategies that will limit host resistance to chemicals and provide long-term control options.

References—Control of Tick Vectors of EP

- Aldridge WN, 1990. An assessment of the toxicological properties of pyrethroids and their neurotoxicity. *Toxicology* 21(2):89–104.
- Almazán C, Lagunes R, Villar M, Canales M, Rosario-Cruz R, Jongejan F, de la Fuente J, 2010. Identification and characterization of *Rhipicephalus (Boophilus) microplus* candidate protective antigens for the control of cattle tick infestations. *Parasitol Res* 106(2):471–479.
- Anonymous, 1994. Pesticide information profile: pyrethrins. Cornell University, Extension Toxicology Network. Available at: <http://pmep.cce.cornell.edu/profiles/extoxnet/pyrethrins-ziram/pyrethrins-ext.html>
- Campbell WC, Benz GW, 1984. Ivermectin: a review of efficacy and safety. *Vet Pharmacol Therap* 7:1–16.
- Canales M, Almazán C, Naranjo V, Jongejan F, de la Fuente J, 2009. Vaccination with recombinant *Boophilus annulatus* Bm86 ortholog protein, Ba86, protects cattle against *B. annulatus* and *B. microplus* infestations. *BMC Biotechnol* 9:29.
- de la Fuente J, Almazán C, Canales M, de la Lastra JMP, Kocan KM, Willadsen P, 2007. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. *An Hlth Res Rev* 8:23–28.
- de la Fuente J, García-García JC, González DM, Izquierdo G, Ochagavía ME, 2000b. Molecular analysis of *Boophilus* spp. (Acari: Ixodidae) tick strains. *Vet Parasitol* 92:209–222.
- de la Fuente J, Kocan KM, 2006. Strategies for development of vaccines for control of ixodid tick species. *Parasit Immunol* 28:275–283.
- de la Fuente J, Rodríguez M, García-García JC, 2000a. Immunological control of ticks through vaccination with *Boophilus microplus* gut antigens. *Ann NY Acad Sci* 916:617–621.
- de la Fuente J, Rodríguez M, Montero C, Redondo M, García-García JC, Méndez L, Serrano E, Valdés M, Enriquez A, Canales M, Ramos E, Boué O, Machado H, Lleonart R, 1999. Vaccination against ticks (*Boophilus* spp.): the experience with the Bm86-based vaccine Gavac™. *Genet Anal-Biomol E* 15:143–148.
- de la Fuente J, Rodríguez M, Redondo M, Montero C, García-García JC, Méndez L, Serrano E, Valdés M, Enriquez A, Canales M, Ramos E, de Armas CA, Rey S, Rodríguez JL, Ariles M, García L, 1998. Field studies and cost-effectiveness analysis of vaccination with Gavac™ against the cattle tick *Boophilus microplus*. *Vaccine* 16:366–373.
- de Vos S, Zeinstra L, Taoufik O, Willadsen P, Jongejan F, 2001. Evidence for the utility of the Bm86 antigen from *Boophilus microplus* in vaccination against other tick species. *Exp Appl Acarol* 25:245–261.
- Dryden MW, Payne PA, 2004. Biology and control of ticks infesting dogs and cats in North America. *Vet Therap* 5(2):139–154.
- Drummond RO, 1985. Effectiveness of Ivermectin for control of arthropod pests of livestock. *Southwest Entomol. Suppl* 7:34–42.
- EPA docket number: EPA-HQ-OPP-2008-0023.
- EPA Document, 2007. Reregistration eligibility decision (RED) for permethrin. EPA 738-R-06-017, 202 pp. Available at: <http://www.epa.gov/pesticides/reregistration/permethrin/>
- Fragoso H, Hoshmand Rad P, Ortiz M, Rodríguez M, Redondo M, Herrera L, de la Fuente J, 1998. Protection against *Boophilus annulatus* infestations in cattle vaccinated with the *B. microplus* Bm86-containing vaccine Gavac Vaccine 16:1990–1992.
- García-García JC, González IL, González DM, Valdés M, Méndez L, Lamberti J, D'Agostino B, Citroni D, Fragoso H, Ortiz M, Rodríguez M, de la Fuente J, 1999. Sequence variations in the *Boophilus microplus* Bm86 locus and implications for immunoprotection in cattle vaccinated with this antigen. *Exp Appl Acarol* 23:883–895.
- García-García JC, Montero C, Redondo M, Vargas M, Canales M, Boué O, Rodríguez M, Joglar M, Machado H, González IL, Valdés M, Méndez L, de la Fuente J, 2000. Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. *Vaccine* 18:2275–2287.
- Gough JM, Kemp DH, 1993. Localization of a low abundance membrane protein (Bm86) on the gut cells of the cattle tick *Boophilus microplus* by immunogold plating. *J Parasitol* 79:900–907.

- Graf J-F, Gogolewski R, Leach-Bing N, Sabatini GA, Molento MB, Bordin EL, Arantes GJ, 2005. Tick control: an industry point of view. *Parasitology* 129(S1):S427–S442.
- Hope M, Jiang X, Gough J, Cadogan L, Josh P, Jonsson N, Willadsen P, 2010. Experimental vaccination of sheep and cattle against tick infestation using recombinant 5'-nucleotidase. *Parasite Immunol* 32:135–142.
- Jongejan F, Ulenberg G, 1994. Tick and control methods. *Rev Sci Tech OIE* 4:1201-1226.
- Labruna MB, Kerber CE, Ferreira F, Faccini JLH, de Waal DT, Gennari SM, 2001. Risk factors to tick infestations and their occurrence on horses in the state of São Paulo, Brazil. *Vet Parasitol* 97:1–14.
- Lopes CML, Leite RC, Labruna MB, de Oliveira PR, Borges LMF, Rodrigues de Carvalho HA, de Freitas CMV, Júnior CR, 1998. Host specificity of *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) with comments on the drop-off rhythm. *Mem Inst Oswaldo Cruz* (3):347–351.
- Kemp DH, Pearson RD, Gough JM, Willadsen P, 1989. Vaccination against *Boophilus microplus*: localisation of antigens on tick gut cells and their interaction with the host immune system. *Exp Appl Acarol* 7:43–58.
- Mulenga A, Sugimoto C, Onuma M, 2000. Issues in tick vaccine development: identification and characterization of potential candidate vaccine antigens. *Microbes Infect* 11:1353–1361.
- Moyo B, Masika PJ, 2009. Tick control methods used by resource-limited farmers and the effect of ticks on cattle in rural areas of Eastern Cape Province, South Africa. *Trop Anim Health Prod* 41:517–523.
- Needham GR, 1985. Evaluation of five popular methods for tick removal. *Pediatrics* 75(6):997–1002.
- Rand KN, Moore T, Sriskantha A, Spring K, Tellam R, Willadsen P, Cobon GS, 1989. Cloning and expression of a protective antigen from the cattle tick *Boophilus microplus*. *P Natl Acad Sci USA* 86:9657–9661.
- Rodríguez M, Penichet ML, Mouris AE, Labarta V, Lorenzo Luaces L, Rubiera R, Cordoves C, Sanchez PA, Ramos E, Soto A, Canales M, Palenzuela D, Triguero A, Leonart R, Herrera L, de la Fuente J, 1995. Control of *Boophilus microplus* populations in grazing cattle vaccinated with a recombinant Bm86 antigen preparation. *Vet Parasitol* 57:339–349.
- Rodríguez Valle M, Méndez L, Valdez M, Redondo M, Espinosa CM, Vargas M, Cruz RL, Barrios HP, Seoane G, Ramírez ES, Boué O, Vigil JL, Machado H, Nordelo CB, Piñeiro MJ, 2004. Integrated control of *Boophilus microplus* ticks in Cuba based on vaccination with the anti-tick vaccine Gavac. *Exp Appl Acarol* 34:375–382.
- Sossai S, Peconick AP, Sales-Junior PA, Marcelino FC, Vargas MI, Neves ES, Patarroyo JH, 2005. Polymorphism of the bm86 gene in South American strains of the cattle tick *Boophilus microplus*. *Exp Appl Acarol* (37):199–214.
- Stafford KC, 2007. Tick management Handbook. New Haven, CT, The Connecticut Agricultural Experiment Station, Bulletin 1010.
- Staudinger H, Ruzieka L, 1924. Insektentötende stoffe. I-IV and VIII-X. *Helv Chim Acta* 7:177–458.
- Steward RL Jr, Burgdorfer W, Needham GR, 1998. Evaluation of three commercial tick removal tools. Columbus, OH, The Ohio State University Acarology Laboratory. Available at: ([http://pinnacle.allenpress.com/doi/abs/10.1580/1080-6032\(1998\)009%5B0137:EOTCTR%5D2.3.CO%3B2](http://pinnacle.allenpress.com/doi/abs/10.1580/1080-6032(1998)009%5B0137:EOTCTR%5D2.3.CO%3B2)).
- USDA-APHIS, 2008. Assessment of change in risk for release of *Ehrlichia ruminantium* (heartwater) into the continental United States following discontinuation of the tropical bont tick (*Amblyomma variegatum*) eradication program on St. Croix, U.S. Virgin Islands. Memorandum 556.1.
- USDA-APHIS, 2003. Veterinary Services policy for the quarantine and testing of horses imported into the United States for special events. Memorandum 591.59.
- Wagner SL, 2000. Fatal asthma in a child after use of an animal shampoo containing pyrethrin. *West J Med* 173(2):86–87.
- Willadsen P, 2004. Anti-tick vaccines. *Parasitology* 129(S1):S367–S387.
- Willadsen P, 2006. Tick control: thoughts on a research agenda. *Vet Parasitol* 138:161–168.
- Willadsen P, 2008. Anti-tick vaccines. *Ticks Biology, Disease and Control*. Cambridge: Cambridge University Press, 424–446.
- Willadsen P, Bird P, Cobon GS, Hungerford J, 1995. Commercialization of a recombinant vaccine against *Boophilus microplus*. *Parasitol* 110:843–850.
- Willadsen P, Riding GA, McKenna RV, Kemp DH, Tellam RL, Nielsen JN, Lahnstein J, Cobon GS, Gough JM, 1989. Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*. *J Immunol* 143:1346–1351.

an equivalent total daily dose with a three times daily dosage interval.

* ROUTES OF ADMINISTRATION

The route of administration is the means by which the drug is given and is the third component of the dosage information (dose, dosage interval, route of administration). Even if the correct dose is administered at the appropriate dosage interval, the amount of drug that reaches the target tissues in the body can be significantly altered if an inappropriate route of administration is used.

Routes of administration are identified by where the drug is placed. Drugs given by injection are said to be parenterally administered. Parenteral translates to "beside, beyond, or apart from the intestines" and refers to the space between the intestinal tract and the surface of the skin. As shown in Figure 3-3, *parenteral administration* of drugs is further divided into specific routes based on where the needle

is introduced. *Intravenous (IV)* administration means the drug is injected directly into a vein. IV injections can be given as a single, large volume at one time, called an *intravenous bolus*, or slowly injected or "dripped" into a vein over a period of several seconds, minutes, or even hours as an *intravenous infusion*. As shown in Figure 3-4 a constant-rate IV infusion results in a steady accumulation of drug concentrations in the body until the drug concentrations reach a plateau, or *steady state*, and remain there until the infusion is stopped. An IV bolus is comparable to dumping all the required water into the empty bucket at once, whereas an IV infusion is comparable to dribbling water into the bucket at a faster rate than it leaks out.

IV administration can be used to safely administer a drug that would be irritating or painful if injected into the muscle or beneath the skin. The accidental injection of an IV-administered drug outside the blood vessel is called an *extravascular injection* or *perivascular injection* because

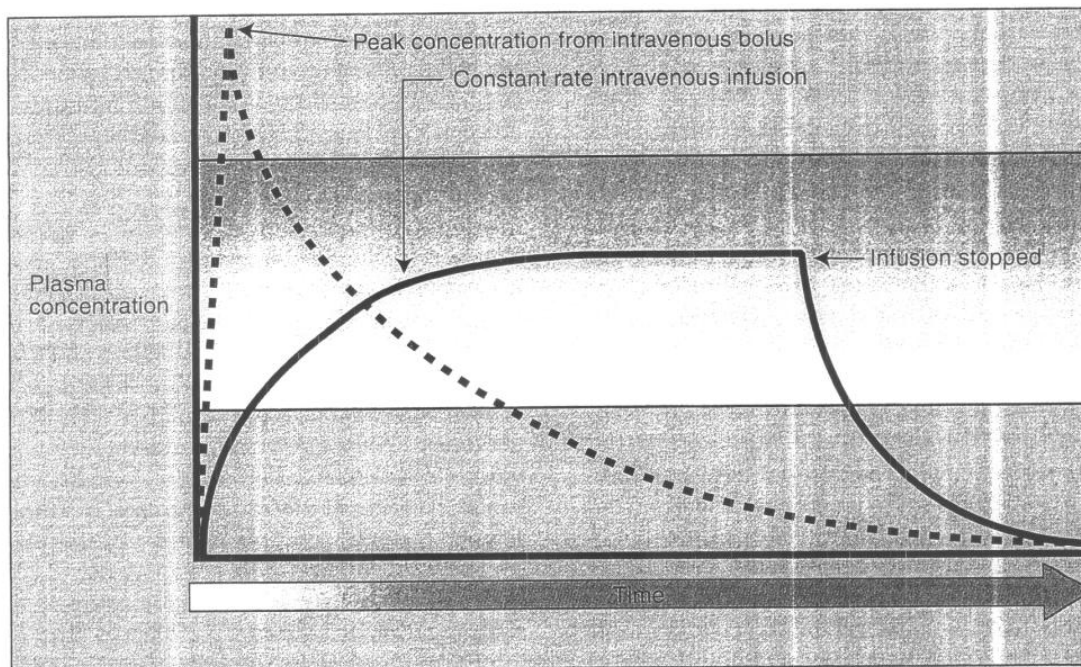


Figure 3-3 Plasma concentrations of a drug after IV bolus injection and infusion. Color shows the therapeutic range.

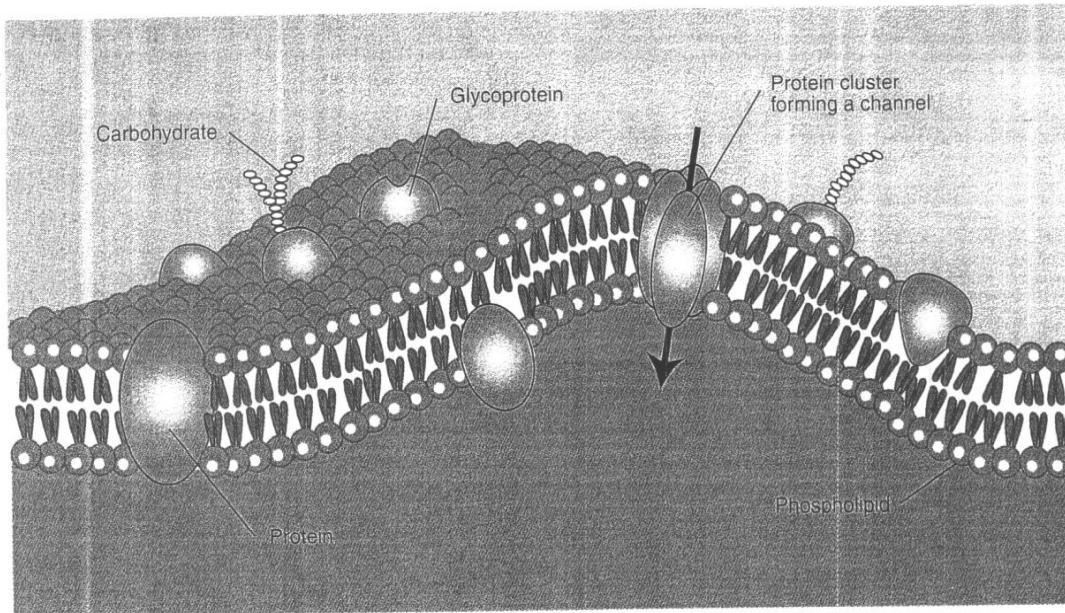


Figure 3-4 Cellular membrane structure.

it is outside (extra-) or around (peri-) the blood vessel. Some drugs injected extravascularly can cause extreme tissue inflammation and tissue necrosis (tissue death).

Although an *intraarterial injection* places the drug directly into the blood vessel, intraarterial administration of a drug produces quite different effects from the same drug given intravenously. Drugs given by the intraarterial route are injected into an artery, not a vein; thus the blood carries the injected drug away from the heart and toward a specific tissue or organ. By contrast, a drug injected intravenously is carried from the tissues toward the heart where it is diluted by the venous blood returning to the heart from the body. By the time an IV drug passes through the heart and the lungs and returns to the tissues, it has been well mixed and diluted in the blood. Unfortunately, an intraarterial injection results in the entire drug being delivered at high concentrations directly to the tissues supplied by that artery. The tissues supplied by this artery would receive drug concentrations that would far exceed the

normal therapeutic range and produce local toxicosis.

Intramuscular administration is another example of the parenteral route of administration. The prefix *intra-* means *within*, so an intramuscularly administered medication is put within the muscle. *Subcutaneous (SC or SQ)* injections are administered "beneath the skin," but not so deep as to be injected into the underlying muscle. *Intradermal (ID) injections* are administered within (intra-), not beneath, the skin with very small needles. The intradermal route is usually reserved for skin testing procedures, such as testing for tuberculosis or reaction to allergenic substances. *Intraperitoneal (IP) injections* are administered into the abdominal body cavity and are frequently used when IV or *intramuscular (IM) injections* are not practical (as in some laboratory animals) or when large volumes of solution must be administered for rapid *absorption*.

Drugs given by mouth are given *per os (PO)*, meaning "via a body opening." Drugs that are applied to the surface of the skin such as lotions and liniments are *topically administered drugs*.

Aerosol administration, meaning “air or gas solution,” indicates that the drug is administered in an inhaled mist or gas and absorbed within the lung airways.

Drugs given by each route of administration described above will be absorbed at a different speed and efficiency. In veterinary medicine the different characteristics of the route of administration provide the appropriate amount of drug to the correct part of the body. Because each route of administration can also be affected by disease in a different way, the veterinary technician must know these patterns to identify potential problems with a route of administration and take appropriate measures to compensate for any problems. These clinical challenges are further discussed below.

MOVEMENT OF DRUG MOLECULES

Part of the reason different routes of administration result in varied drug concentration curves is because of the way drug molecules move from one site to another by different mechanisms. Drug molecules move from point A to point B by four different mechanisms: *passive diffusion*, *facilitated diffusion*, *active transport*, and *pinocytosis* and *phagocytosis*.

PASSIVE DIFFUSION

The majority of drug movement through tissue fluid or membrane barriers is by passive diffusion.

Passive diffusion is the random movement of drug molecules from an area of high concentration to an area of lower concentration, or down the *concentration gradient*. For example, when a colored liquid is poured into a glass of water, the color spreads, or diffuses, to all parts of the water. Similarly, when a drug is injected in the body, it passively diffuses down the concentration gradient from the injection site to the surrounding areas of lower concentrations, eventually reaching a blood capillary and entering the systemic circulation. In this

process no active cellular energy is expended by the body to direct the movement of drug molecules; hence the term passive diffusion.

The concept of diffusion of substances throughout liquid is readily grasped because it is observed in everyday life (e.g., mixing sugar in iced tea, adding milk to coffee). Diffusion is also involved in the movement of drug molecules through cell membranes. For a drug to diffuse from one side of a biologic membrane to the other, the drug must dissolve in the membrane. Cell membranes, on a molecular level, are not solid structures (Figure 3-4). The cell membranes are composed primarily of phospholipids (a molecule with phosphate groups and fatty acid components). These phospholipids give the cell membrane the characteristics of fat. Therefore, for a drug molecule to diffuse from one side of the cell membrane to the other, it must be capable of dissolving in fat or oils. In such cases, the drug molecule passes from the side of the cell membrane with a high concentration of drug molecules to the side with the lower concentration by passive diffusion and without any expenditure of cellular energy.

Whether passive diffusion occurs within a liquid or across a cellular membrane, the drug molecules continue to pass from an area of higher concentration to an area of lower concentration until the drug molecules are equally distributed. In other words, at *equilibrium* the number of molecules at point A are the same as they are at point B. In actuality, once equilibrium is reached, diffusion of the molecules does not stop. The molecules are still moving around, but no net direction of diffusion can be detected because the number of molecules moving from point A to point B is the same as the number moving from point B to point A.

FACILITATED DIFFUSION

Facilitated diffusion is a passive transport mechanism across cell membranes that involves a special “carrier molecule” located in the cellular membrane. The carrier molecule, usually a protein floating among the

Drug Control in Horse Racing in the U.S.

Scot Waterman, DVM
Executive Director
Racing Medication & Testing Consortium

Terminology

Testing Basics

- Every winner of every race in the US goes to the test barn
- Depending on the state, one or two additional horses may be selected for testing
- Typically both urine and blood samples are drawn at the test barn
- Samples are tagged with a unique identifier and sealed to begin a chain of custody

Definitions

- **Screening test**- rapid and sensitive, specificity not crucial. A sample with a positive screening test would be said to be "suspicious"
- **Confirmation test**- sensitive and specific, if the result is positive the presence of a forbidden substance is said to be confirmed.

Definitions

- **Limit of Detection**—the lowest concentration of a drug that can be detected by a particular laboratory method.
- **Limit of Quantitation**—the lowest concentration of a drug that can be reliably quantified by a particular laboratory method. Generally higher than the LOD.

Definitions

- **Withdrawal Time** – the time period that must elapse from the time of administration of the last dose of a drug formulation until the drug concentration decreases below some concentration in urine and/or blood.
 - That concentration may be the LOD, the LOQ, or some predetermined concentration.
 - Factors affecting withdrawal time include dose, route of administration, formulation, number of doses administered, accuracy and precision of the assay, and within-horse and between-horse variability among other factors

5

LD3

#7 *

Definitions

- **Regulatory Limit**
 - When a drug and/or its metabolites are found in the urine or blood at a concentration that is at or below the regulatory limit, no administrative action is taken.
 - Also called a "threshold" or "decision level"
 - European terminology = "screening limit"
 - Can be a LOD, LOQ, arbitrary, based on data from administration studies

Definitions

- **Zero Tolerance**—somewhat of a myth since no testing methodology can detect down to a concentration of zero.
 - Usually suggests that the laboratory calls positive any concentration of drug that is detected
 - Entirely dependent on the method being used though

Zero tolerance?

Screening Method	Limit of Detection	Detection Time
Thin Layer Chromatography	~100 ng/ml	~24 hours
ELISA	~1 ng/ml	~72-96 hours
Mass Spectral Methods	~25 pg/ml	~7-30 days

Zero tolerance?

	Screening Method	Sample contains 150 ng/ml	Sample contains 50 ng/ml	Sample contains 50 pg/ml
State A	Thin Layer Chromatography	Positive	Negative	Negative
State B	ELISA	Positive	Positive	Negative
State C	Mass Spectral Methods	Positive	Positive	Positive

Definitions

- **Hay, oats and water**—there is really no definition since the phrase means different things to different people.
 - No drug administration on raceday?
 - No detectable concentrations of drugs on raceday? Since we can't detect down to zero whose definition of "detectable" is used
 - No drugs ever?

Hay, Oats and Water

- Keene Dangerfield, The Blood-Horse, 1979:

"One things I will say is that people who are talking about *going back* to racing on hay, oats and water don't know what they are talking about. We've *never* raced on hay, oats and water...Today we simply are able to find out what they are medicating them with."

U.S. Regulatory Framework

U.S. Regulatory Framework

- State statute enables a state agency to govern pari-mutuel wagering
- Commission has power to write, enforce and adjudicate rules...executive, quasi-judicial, quasi-legislative
- Commissioners are usually appointed by the governor
- Reason...the industry asked for it to eliminate bookmaking competition, state wanted to control since gambling is a vice

U.S. Regulatory Framework

- Makeup of commission varies state-by-state
 - Some states do not allow those involved in the industry day-to-day to serve as commissioners
 - Number of commissioners (as low as 1, as high as 13)
- Funding for operations varies state-by-state
 - Majority of states funded through general funds but some based on revenue sources from handle
- Rulemaking process varies state-by-state
 - NJ, NY, CA from start to finish process can take over one year. Some states also have regulations encoded in statute...need to involve legislature to change

U.S. Regulatory Framework—Challenges

- Commission only quasi-legislative
 - Generally some state legislature oversight which creates another potential hurdle for model rules
- Commission only quasi-judicial
 - Violations appealed to state court which historically have reduced significant penalties
- Funding, funding, funding
- Horsemen/practicing veterinarians have too much influence in some states

U.S. Regulatory Framework—What actually happens

- RMTC develops a model rule on a therapeutic medication
- RCI Model Rules Committee and Board of Directors approve model rule
- The adoption of the model rule will be more restrictive than what is allowed under state "A" current rule
- State "A" begins adoption process
- RMTC and sometimes RCI show up at a public commission hearing and present rationale
- Local horsemen and veterinarians say adoption of rule will hurt racing in state "A"
- You are the commissioner...who do you listen to? The guy from out of town or the local guys who say racing in your state will suffer?

Racing Medication & Testing Consortium

RMTC History

- **Formation:**
 - Began with the AAEP Medication Summit, December 2001
 - Summit designed to determine level of consensus on uniform medication policy
 - All organization that participated in original meeting are still involved nine years later
 - Organization has moved beyond rule uniformity as a singular goal
- Incorporated as a 501 (c) (3) charitable organization since 2003

RMTC Board

- Horsemen—THA, HBPA, CTT
- Tracks-Magna, Churchill, Oak Tree, Del Mar, HTA, TRA, NYRA, Keeneland
- Owners/Breeders—TOBA, TOC, KTA
- Veterinarians—AAEP
- Security—TRPB
- Regulatory Association—RCI
- Breed Registries/Other—AQHA, The Jockey Club, NTRA, USTA, Hambeltonian Society, Arabian Jockey Club
- Jockeys—Jockey's Guild

Goals and Objectives

- Uniform medication rules
- Uniform testing procedures
- Uniform thresholds and withdrawal guidelines for therapeutic medications
- Unbiased source of information on medication issues for state racing commissions
- Develop intelligence on new threats to integrity
- Improved security measures
- Better communication regarding medication issues



RMTC Goals and Objectives

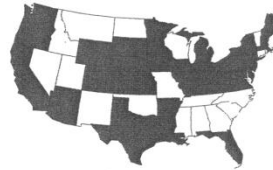
- Generally successful in the development of model medication policies and encouragement of adoption at state level
 - Large board viewed as an "industry consensus" once language is completed
 - Have been able to overcome some of the parochial issues as state level
 - Able represent RMTC in person at state commission meetings when requested
 - Development of a network of individuals within commissions to assist in the adoption process

Model Rules V. 2005

- **Permitted Therapeutics:**
 - IV administration of furosemide 4 hours prior to post
 - Anti-ulcer medications permitted 24 hours prior to post
 - One of three NSAIDs – Bute, Banamine & Ketoprofen – 24 hours prior to post
- **Prohibited Practices**
 - ESWT restrictions
 - Possession of blood doping agents

Salix/NSAID Adoption Progress

- Language adopted
- ▣ Language in the process of being adopted
- Process require legislative action
- Process has not been started
- No horse racing or harness racing commission

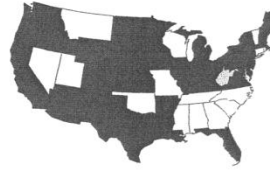


Androgenic Anabolic Steroids

- Unregulated in US (except Iowa) up until 2007
- Model rule developed proactively by RMTC in 2007
- Rule set thresholds for 4 anabolic steroids and prohibits presence of any others
- Adopted the international thresholds for endogenous anabolic steroids
- Thresholds for stanozolol and nandrolone based on the LOD using GC/MS in urine (best science at the time)

AAS Adoption Progress

- Language adopted
- Language in the process of being adopted
- Process pending completed administration studies
- Process has not been started
- No horse racing or harness racing commission



RMTC Goals and Objectives

- Industry closer to uniformity than ever before
- Very few in industry thought RMTC would be this successful in driving uniformity
- Organizations involved standing behind rules
- Challenges still remain:
 - Penalties
 - Difficult to get stewards/commissions to depart from status quo
 - How to make smaller states relevant, part of process
 - Commissions that want to look tougher than anyone else
 - Grinding process

RMTC Goals and Objectives

- Improving Communication Efforts—Successful
 - Industry
 - Presence at conferences and organizational board meeting
 - Respected source of information for industry trades
 - Withdrawal times database on website
 - Regular newsletters, annual report
 - Wagering Public
 - Regular spot on Steve Dyk show on Sirius
 - Educational materials on website
 - Database of positive tests with explanation of drug in development
 - Source for mainstream press

RMTC Goals and Objectives

- Development of Uniform Withdrawal Times and Thresholds—Too Early To Tell
 - Accomplished:
 - Identified drugs with legitimate therapeutic uses
 - Prioritized drugs into five groups based on number of violations over 5 year period
 - Collected previous science on each drug
 - Ongoing:
 - Administration of each drug at relevant doses to 20 horses for maximum statistical power
 - Analyze drug concentrations and determine withdrawal time
- Ultimate goal: uniform withdrawal time with a corresponding concentration of drug which regulates the withdrawal time

Withdrawal Times Research

- | | |
|--------------------|--------------------|
| ■ Priority Group 1 | ■ Priority Group 2 |
| - Acepromazine | - Boldenone |
| - Butorphanol | - Dantrolene |
| - Detomidine | - Dexamethasone |
| - Glycopyrrolate | - Fluphenazine |
| - Lidocaine | - Hydroxyzine |
| - Mepivacaine | - Nandrolone |
| - Methocarbamol | - Stanozolol |
| - Pyrilamine | - Testosterone |

RMTC Goals and Objectives

■ Challenges:

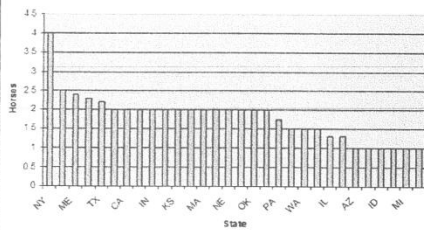
- Has taken much longer than anticipated
 - Good science takes time but frustrating since we wanted answers yesterday
 - No dedicated laboratory to analyze the significant number of samples generated
 - Discrepancies in data between labs due to methodology differences
- How do we determine desired withdrawal time?
 - Pharmacodynamic markers
 - Best educated guess
- How to present data to reach goal of uniformity

RMTC Goals and Objectives

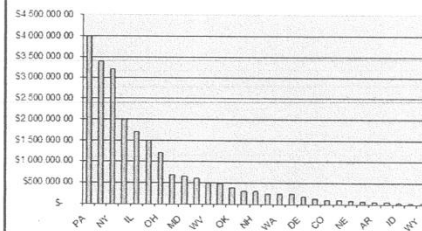
■ Uniform Testing Procedures—Too Early To Tell

- Post-race testing in the United States:
 - Currently 18 laboratories conducting testing in US...too many labs chasing too few dollars
 - Only 5 labs ISO 17025 accredited
 - 30% of samples were tested by an accredited lab in '08
 - Funding is state-by-state and many times is under state procurement laws which reward low bidder or is statutorily directed
 - No industry standards for US testing labs
 - Not enough attention being paid to sample collection strategies

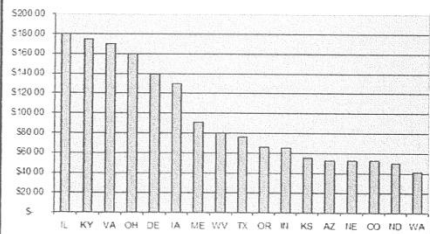
Number of Horses Selected Per Race



Laboratory Budget By State



Per Sample Testing Costs (Combined Blood and Urine)



RMTC Drug Testing Initiatives

- Task Force formed in September 2008
- First meeting...design the best system for US Drug Testing irrespective of funding and political concerns
- Consensus that we should utilize laboratory standards developed by the World Anti-Doping Association where applicable and work towards the development of a performance-based system
- Other related topics discussed...next generation of lab directors, sample selection strategies and the use of frozen samples

RMTC Drug Testing Initiatives

- **Creation of Industry Standards for Labs:**
 - Edited version of the Lab Standards document created by the World Anti-Doping Association has been developed by the committee
 - Standards rely on ISO 17025 accreditation as the first step
 - An external proficiency program is then conducted
 - Labs failing proficiency cannot conduct testing
 - A percentage of the laboratory budget is mandated to be directed towards research
 - Working on a truly external quality assessment program for testing laboratories...hope to have in place during Q2 in 2010

RMTC Drug Testing Initiatives

- **Creation of Industry Standards for Labs (continued):**
 - Result will create the first set of industry standards for post-race testing labs in US
 - Result will create the first ever external QAP in the US
 - States/industry will have a document to "sell" in order to provide incremental funding for lab to meet standards
 - Will need funding for the industry organization that takes on the role of WADA

RMTC Drug Testing Initiatives

- **Possible outcomes**
 - Incremental funding by states in order to allow their labs to meet the standards
 - Reduction/consolidation of testing labs due to inability to meet standards and/or failure in proficiency
- **If implemented will lead to greater uniformity in laboratory procedures and methodologies**

General Industry Challenges

- Funding for RMTC to continue research
- Funding from state for commissions to increase spending on testing, investigatory capabilities, adjudication
- "Silver bullet" thinking on complex issues
- Dealing with issues that may be strictly based on perception and misinformation
- Culture of medication use
- Indirect issues that affect medication use

RMTC: Contact Us

- www.rmtcnet.com



review articles***Plasma or serum in therapeutic drug monitoring and clinical toxicology***

DONALD R.A. UGES

Introduction

In therapeutic drug monitoring and clinical toxicology most determinations of the concentration of a substance are carried out in blood or blood components. Whole blood can only be analysed if the blood has been sampled in collecting tubes containing an anticoagulant. When such samples are centrifuged, plasma is obtained. Therefore, plasma always contains one or more anticoagulants. Heparin sodium (3-100 U/ml) is most widely used for this purpose, but citrate, edetate, oxalate and fluoride are also suitable additives. Serum is the clear liquid that separates from blood when blood is allowed to clot completely and then centrifuged. Therefore serum contains no fibrogen and no anticoagulants.

The choice between whole blood on the one hand and either plasma or serum on the other is clear. Whole blood concentrations are only measured if the compound is concentrated in the erythrocytes (e.g. lead, cyanide, mercury, carbon monoxide, chlor-thalidone),^{1,3} if there is a fluctuating erythrocyte-plasma ratio (ciclosporin A),⁴ or because of the risk of loss during storage or centrifugation.⁵

The clinical effect of several compounds correlates better with the concentration in a tissue compartment than with the serum or plasma concentration. Considering erythrocytes as a readily available tissue, whole blood should be the matrix of choice in therapeutic drug monitoring and toxicology.^{6,7} Sometimes lymphocyte concentration can predict the therapeutic effect better (epirubicin, mitoxantrone).^{8,9} In nearly all other cases, plasma or serum concentrations are measured.

Is there any difference between serum and plasma concentration?

The expression 'plasma concentration' is a part of the title of many articles in the literature. Reading these articles, however, it appears that serum samples have often been taken. The authors very seldom present their arguments for the choice between plasma or serum.

Some of the advantages of plasma over serum are:

- large volume;
- no delayed clotting;
- less risk of haemolysis;
- the sample is often suitable for both whole blood and plasma monitoring.

Some of the disadvantages of plasma over serum are:

- the (unknown) influence of the anticoagulant on the assay, the protein binding and the stability of the sample;
- the (unknown) influence of additives or impurities in the anticoagulant on the assay and the concentration;
- the risk of the formation of small clots (incomplete mixing, instability);
- dilution of the sample;
- sampling is more expensive;
- choice of anticoagulant can be confusing.

ADVANTAGES OF PLASMA OVER SERUM***Larger available volume***

If blood is allowed to clot and is then centrifuged,

Keywords

Anticoagulants
Blood coagulation
Blood preservation
Plasma
Protein binding
Serum
Therapeutic drug monitoring
Toxicology

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Uges DRA. Plasma or serum in therapeutic drug monitoring and clinical toxicology. *Pharm Weekbl [Sci]* 1988;10:185-8.

Abstract

The relative merits of plasma and serum in blood analysis are reviewed. The expression 'plasma concentration' is often used in the literature, although serum samples have been taken. In most cases serum and plasma concentrations of analytes are the same. The choice depends mostly on the policy of the hospital or the availability of the test tubes in the ward. Some of the advantages of plasma over serum are large volume, no delayed clotting, less risk of haemolysis. In addition, the sample is often suitable for both whole blood and plasma monitoring. Some of the disadvantages of plasma over serum are the (unknown) influence of the anticoagulant on the assay, on the protein binding and on the stability of the sample, the (unknown) influence of additives or impurities in the anticoagulants on the assay and on the concentration, the risk of the formation of small clots and dilution of the sample.

about 30 to 50% of the original volume is collected as serum (upper layer). A slightly larger sample (in general 50%), known as plasma, can be obtained by centrifugation of a blood sample collected in a tube containing an anticoagulant. Serum and plasma are not significantly different with respect to drug analysis.

Despite the fact that serum is devoid of the proteins associated with the clotting process, the most important proteins (quantitatively speaking), such as the albumins and globulins, are present in similar amounts (approximately 2% wt/vol) in both serum and plasma. Thus plasma is in general preferred because of its greater yield from blood samples. The greater yield the greater the amount of drug and the fewer the problems with sensitivity, or with sampling neonates.^{10,11}

Delay through time needed for clotting

Clotting can sometimes be of long duration. This is especially true when samples are taken in polypropylene test tubes, in which case clotting can continue even after centrifugation.

Decreased risk of haemolysis

The chlorothalidone concentration in erythrocytes is about 40 times as high as that in plasma.⁸ One percent of haemolysis (which cannot easily be seen) will increase the apparent plasma concentration by 25%. This problem is even more acute when serum samples are produced from whole blood.

The separation by centrifugation should be carried out as quickly as possible in order to prevent any effects due to lysis of the blood clot.¹¹ This phenomenon is well known in the analysis of the endogenous compounds potassium and iron in serum.

Suitability of the sample for both whole blood and plasma analysis

If several analyses have to be carried out on the blood of a single patient, determinations can be carried out in whole blood as well as plasma from one blood sample containing a suitable anticoagulant. In bedside chemistry whole blood is preferred to plasma or serum, because this avoids a centrifugation step.¹²

DISADVANTAGES OF PLASMA IN RELATION TO SERUM

The influence of the anticoagulant on the assay

Walters and Roberts showed that heparin at a concentration of 100 U per ml interfered with the gentamicin EMIT[®] assay, but not with the TD_x[®] FPIA assay.¹³ This effect is thought to be due to the inhibition of enzyme activity rather than disturbance of the antigen-antibody reaction. Blood collected into heparinized tubes usually contains approximately 30 U of heparin per ml and at this level no interference with the EMIT[®] assay occurs. How-

ever, plasma derived from specimens collected from arterial and venous blood is likely to yield erroneous results.

The formation of a gentamicin-heparin complex has been demonstrated by Myers *et al.*¹⁴ Cipolle *et al.* concluded that heparinized tubes should not be used when collecting blood samples for assays where inactivation of gentamicin would give false values.¹⁵ Edetate was for gentamicin a suitable alternative. On the other hand, the Dutch foundation 'Quality control clinical drug analysis and toxicology' (KKGTT) found with its quality control programme that edetate interfered strongly with the FPIA assay and not with the EMIT[®] assay of carbamazepine and with the EMIT[®] assay and not with FPIA assay of valproic acid. The reason of this interference is unknown (Dijkhuis IC, personal communication, 1988).

The influence of the anticoagulant on protein binding

A discrepancy in the measurement of lidocaine in heparinized blood and in serum could be expected, as heparin increases the free fraction of lidocaine.¹⁶ Lidocaine is subsequently redistributed from the plasma into red blood cells.

The percentage of free ibuprofen in heparinized plasma is significantly higher than in non-heparinized plasma.¹⁷ Although small doses of heparin can affect drug binding, the extent and variability of the effect depends on the biological activity of the heparin, and varies with the manufacturer and batch, time of sampling, and food intake.¹⁸ Although the intravenous administration of heparin results in a high lipolytic activity *in vivo* and consequently increases the concentrations of non-esterified fatty acids *in vitro*, it is unlikely that this will happen after adding heparin to the tube.

Non-esterified fatty acid can displace numerous drugs from their binding sites in plasma. When the non-esterified fatty acids concentration increases *in vitro*, phenytoin will be displaced from the binding sites. The free phenytoin then shifts from the plasma into the erythrocytes, resulting in a lower phenytoin plasma level (up to 20% below its original value!).¹⁹ The same phenomenon was found with amitriptyline, imipramine and maprotiline.⁶ The recovery of chlorpromazine added to heparinized plasma *in vitro* was reduced compared with that obtained with water or oxalated plasma.²⁰

The influence of the anticoagulant on the stability of the sample

When heparin deteriorates or adsorbs onto the wall of the collecting tube, clotting will still occur in a clear plasma sample. The pH of the sample can be changed by using sodium citrate, or oxalate as anticoagulant. This change of pH may influence the stability of the drug in the sample (*e.g.* atracurium). Sodium fluoride can be used as anticoagulant (cal-

cium binder), and as a preservative (antimicrobial) of ethanol. Blood samples that are to be tested for ethanol must contain 10 mg sodium fluoride per ml during storage.^{21,22}

The influence of additives or impurities in the anticoagulant on the assay

Benzyl alcohol, which may be added to heparin as a preservative, can disturb the fluorimetric assay of corticosteroids.²³ Serum zinc concentrations are 16% higher than plasma concentrations. This phenomenon was explained by a release of zinc from platelets during coagulation of the blood samples.²⁴ Keyzer *et al.*, however, found no difference between the serum and plasma zinc levels of fifty volunteers.²⁵

The influence of additives or impurities in the anticoagulant on the concentration

Anticoagulants can contain impurities which are precisely the compounds to be determined, *e.g.* lead, aluminium, copper, fluoride.

The risk of clot formation because of poor mixing or poor stability

Unfortunately, blood samples in heparinized tubes are sometimes not very well mixed. In such cases a partially clotted sample will arrive in the laboratory.

Improper dilution of the sample

We have had a number of incidents in which blood samples of about 5 ml were diluted with about 1 ml of a solution of anticoagulant (20% dilution!). Differences in serum and plasma transcobalamin II levels in the literature have been explained by dilution by EDTA or sodium fluoride solutions.²⁶

Cost

Sample tubes with heparin are approximately 10% more expensive than non-heparinized tubes.

The choice of anticoagulant

There are several anticoagulants, each of which must be used in a different concentration. Examples are sodium heparin, lithium heparin, potassium edetate, sodium edetate, sodium citrate, and sodium fluoride. The differences in the influence of these anticoagulants on the various assays are largely unknown.

Differences were demonstrated between serum and plasma levels of endogenous compounds, which may be of sufficient magnitude to alter clinical interpretation of some results when using different radioassay procedures and different anticoagulants.²⁷ These differences are in general larger with immunoassays than with chromatographic methods. Cholinesterase activity can be determined in serum or heparinized plasma. Sodium fluoride and citrate should not be used as anticoagulants because they

depress cholinesterase activity as measured by several methods.²⁸

Anticoagulants used in sampling plasma can confound diffusion assays by causing alterations in the agar gel matrix. Therefore, in the microbiological measurement of, for example, erythromycin, cytarabine or 5-fluorouracil the use of serum is recommended.²⁹

Conclusion

In conclusion, we can say that in most cases serum and plasma analyte concentrations are the same. The choice depends mostly on the established practice in the hospital or the kind of available test tubes in the ward. In special cases such as the determination of free drug levels, however, there are important differences. It is advisable, therefore, that during kinetic studies of drugs, the similarity of serum and plasma levels for the compound being studied be proven. Furthermore, to avoid confusion, authors should be careful and precise in their presentations so that readers are aware that blood, plasma or serum is the sample under discussion.

References

- Casarett LJ, Doull J. Toxicology. The basic science of poisons. New York: Macmillan Publishing Company, 1975.
- Moffat AC, ed. Clarke's isolation and identification of drugs. 2nd ed. London: The Pharmaceutical Press, 1986.
- Beermann B, Groschinsky-Grind M. Clinical pharmacokinetics of diuretics. *Clin Pharmacokinet* 1980;5:221-45.
- Keown PA, Stiller CR, Stawecki M, McMichael J, Howson W. Pharmacokinetics and interactions of ciclosporin. In: Schindler R, ed. Ciclosporin in autoimmune diseases. Berlin: Springer-Verlag, 1985:39-42.
- Uges DRA, Van der Paauw HJW. Alcohol, de bloedproef en de contra-expertise [Alcohol, blood test and counterexpertise]. *Ned Forens Tijdschr* 1982;2:7-9.
- Maguire KP, Burrows GD, Norman JR, Scoggings BA. Blood plasma distribution ratios of psychotropic drugs. *Clin Chem* 1980;26:1624-5.
- Linnoila M, Dorrity F, Jobson K. Plasma and erythrocyte levels of tricyclic antidepressants in depressed patients. *Am J Psychiatry* 1978;135:557-61.
- De Vries EGE, Greidanus J, Mulder NH, et al. A phase I pharmacokinetic study with 21-day continuous infusion of epirubicin. *J Clin Oncol* 1987;5:1445-51.
- De Vries EGE, Greidanus J, Mulder NH, et al. A phase I and pharmacokinetic study with 21-day continuous infusion of mitoxantrone (MX). *Am Soc Clin Oncol* 1988;7:58.
- Smits RV, Stewart JT. Textbook of biopharmaceutic analysis. Philadelphia: Lea & Febiger, 1981:3-16.
- Toseland PA. Samples and sampling. In: Moffat AC, ed. Clarke's isolation and identification of drugs. 2nd ed. London: The Pharmaceutical Press, 1986:111-7.
- Hichs JA, Iosefsohn M. Another physician's office analyser: the Abbott vision evaluated. *Clin Chem* 1987;33:817-9.
- Walters MI, Roberts WH. Gentamicin/heparin interactions: effects on two immunoassays and on protein

- binding. *Ther Drug Monitor* 1984;6:199-202.
- ¹⁴ Meyers DR, DeFehr J, Bennett WM, Porter GA, Olsen GD. Gentamicin binding to serum and plasma proteins. *Clin Pharmacol Ther* 1978;23:356-60.
- ¹⁵ Cipolle RJ, Zaske DE, Crossley K. Gentamicin/tobramycin. Therapeutic use and serum concentration monitoring. In: Taylor WJ, Finn LA, eds. *Individualizing drug therapy. I*. New York: Frank Cross Townsend Inc., 1981:138.
- ¹⁶ Dube LM, Ho-Ngoc A, Davies RF, Beaulands DS, Mousseau N, McGilveray IJ. Influence of protamine on heparine induced increases of lidocaine free fraction. *Res Commun Chem Pathol Pharmacol* 1983;42:401-15.
- ¹⁷ Pearce GA, Brown KF. Heat inhibition of *in vitro* lipolysis and ¹⁴C ibuprofen protein binding in plasma from heparinized uraemic subjects. *Life Sci* 1983;33:1457-66.
- ¹⁸ Naranjo CA, Sellers EM, Khow V, Alexander P, Fan T, Shaw J. Variability in heparin effect on serum drug binding. *Clin Pharmacol Ther* 1980;28:545-50.
- ¹⁹ Schulz P, Abang A, Giacomini JC, Blaschke TF, Giacomini KM. Effect of heparin on the red blood cell-to-plasma concentration ratio of diphenylhydantoin and prazosin. *Ther Drug Monitor* 1983;5:497-9.
- ²⁰ Whelpton R. Tricyclic antidepressants and neuroleptics. In: Curry AS, ed. *Analytical methods in human toxicology. Part I*. Weinheim: Verlag Chemie, 1985:139-58.
- ²¹ Flanagan RJ, Widdop B. Clinical toxicology. In: Curry AS, ed. *Analytical methods in human toxicology. Part I*. Weinheim: Verlag Chemie, 1985:37-66.
- ²² Froentjes W, Schute JB, Strengers Th, Verwey AMA. Analytisch-chemische aspecten van de wijziging van de Wegenverkeerswet (II) [Analytical and clinical aspects of the change in the Traffic Act (II)]. *Pharm Weekbl* 1976;111:349-62.
- ²³ Werk EE jr. Interference of heparin containing benzyl alcohol in the fluorometric determination of plasma corticosteroids. *J Clin Endocrinol* 1967;27:1350.
- ²⁴ Foley B, Johnson SA, Hackley B, Smith JC jr, Halsted JA. Zinc content of human platelets. *Proc Soc Exp Biol Med* 1968;128:265-9.
- ²⁵ Keyzer JJ, Oosting E, Wolthers BG, Muskiet FAJ, Hindriks FR, Van der Slik W. Zinc in plasma and serum: influence of contamination due to the collection tubes. *Pharm Weekbl [Sci]* 1983;5:248-51.
- ²⁶ Carmel R. Vitamin B12-binding proteins in serum and plasma in various disorders. Effects of anticoagulants. *Am J Pathol* 1978;69:319-25.
- ²⁷ Kubasik NP, Sine HE. Results for serum and plasma compared in 15 selected radioassays. *Clin Chem* 1978;24:137-9.
- ²⁸ Huizinga JR, Gips CH. Evaluation of the UV-340 spectrophotometric determination for pseudocholinesterase activity (EC 3.1.1.8) in human serum. *J Clin Chem Clin Biochem* 1987;25:161-5.
- ²⁹ Smits RV, Stewart JT. Microbiologic assay methods. In: *Textbook of biopharmaceutic analysis*. Philadelphia: Lea & Febiger, 1981:249-59.

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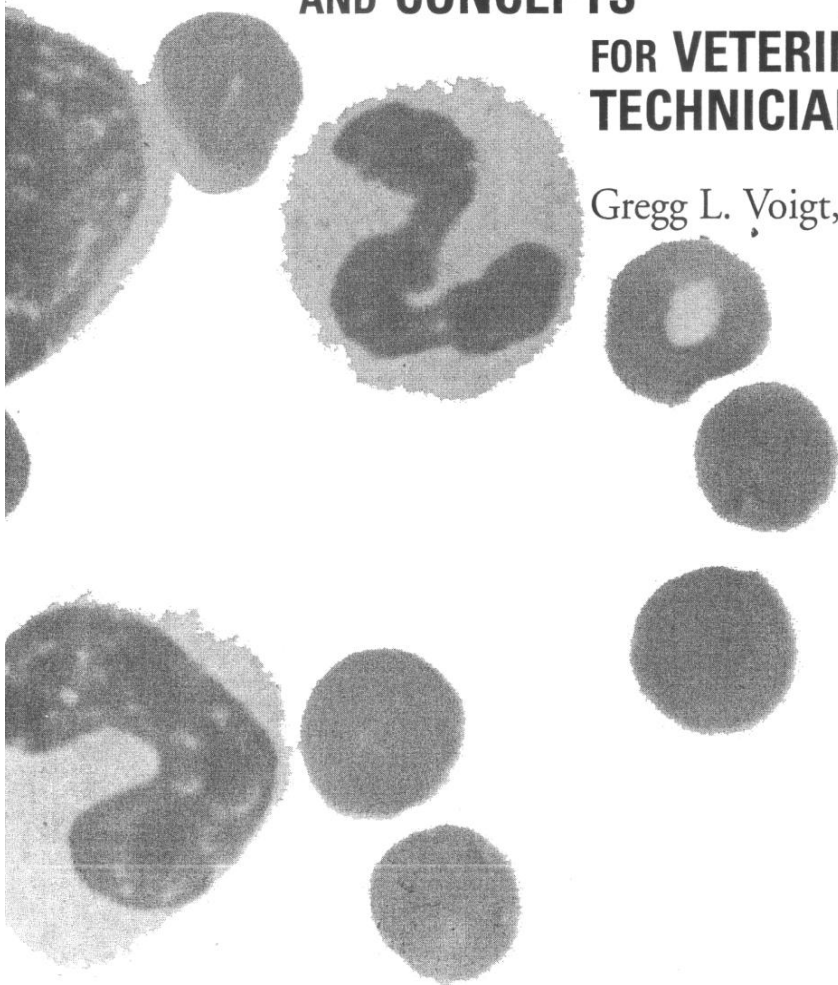
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**HEMATOLOGY TECHNIQUES
AND CONCEPTS**

**FOR VETERINARY
TECHNICIANS**

Gregg L. Voigt, DVM





CHAPTER 2

Blood Composition

Blood is a type of connective tissue, and collecting a blood sample is essentially taking a tissue biopsy. Blood is composed of various cells surrounded by a noncellular substance, just like other tissues such as fibrous tissue, bone, or cartilage. The major difference is, of course, that the extracellular substance in blood is a liquid, called plasma. This characteristic, plus the fact that much of this “tissue” is located near the surface of the animal, make collecting a sample of blood comparatively easier than sampling more solid organs and tissues.

PCV, Buffy Coat, Plasma

If drawn blood is kept from clotting by the addition of an anticoagulant (see Chap. 4), we can separate the blood into its various components. If allowed to set undisturbed (or placed in a centrifuge to speed up the separation of components), three distinct layers will appear: the heavy red cells on the bottom, the lighter white cells and platelets (buffy coat) in the middle, and the plasma (liquid) on top (Fig. 2.1).

The bottom one-third to one-half of the tube will be dark red and will contain the heaviest (most dense) structures, the erythrocytes (red blood cells). The measurement of the percentage of these cells in the blood is called the **packed cell volume (PCV)** or **hematocrit (Hct)** and is commonly one of the first blood evaluations performed (see Chap. 6 and Plate 1).

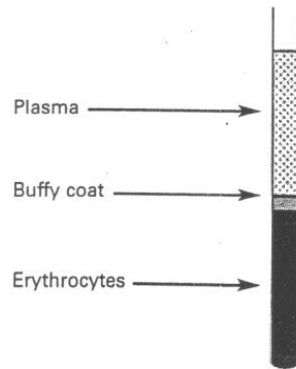


Fig. 2.1 The components of unclotted blood.

Directly on top of the red blood cells will be a relatively thin, white to tan layer called the buffy coat. This layer contains the **leukocytes** (white blood cells) and the **platelets** (important in blood clotting). The thickness of this layer can be the basis for a rough estimate of the relative numbers of these cells in the sample. The occasional appearance of a light pink to red color in the buffy coat indicates the entrapment of some lighter density erythrocytes in this layer.

At the top of the tube will be the fluid portion of the blood sample, called **plasma** since the blood has been separated without clotting. If the blood had been allowed to clot, several proteins in the fluid would have been used to form the clot, and the fluid would then be called serum (Fig. 2.2). The plasma color will vary from clear to straw to yellow-orange (due to species, diet, and physiologic or pathologic conditions) but should

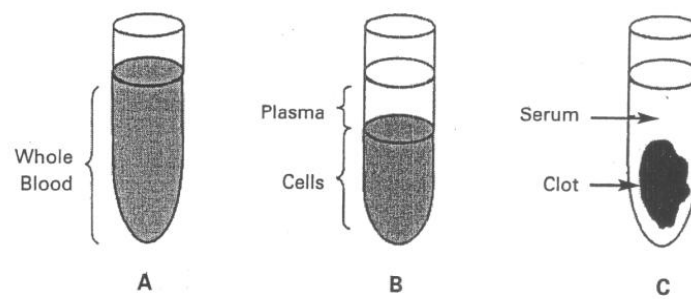


Fig. 2.2 Physical difference between plasma and serum. (A) Mixed fluid and cells in whole blood. (B) With anticoagulant added, cells separate from plasma. (C) Without an anticoagulant, cells and clotting factors separate from serum.

be transparent. The technician should always note the visual characteristics of the plasma, especially any cloudiness and abnormal coloration or layering, and write descriptive comments on the laboratory report. (Remember, the technician may be the only one to see the sample and should pass on information!)

Blood Cells

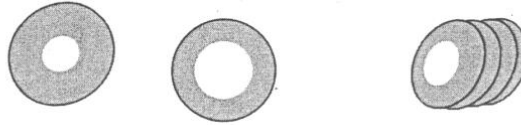
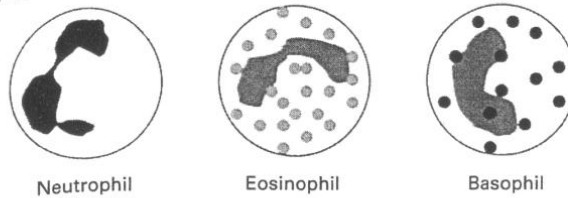
In order to see and evaluate individual blood cells, a drop of blood must be spread on a slide and stained. This is one of the most common and important hematology procedures. Techniques for making and staining the blood smear, as well as suggested methods of viewing the slide under the microscope to ensure optimum examination of the cells, are given in Chapters 5 and 6. There are seven “formed elements” (cells or cell fragments) found in the blood:

Erythrocytes (red blood cells, or RBCs)	(1)
Leukocytes (white blood cells, or WBCs)	
Granulocytes	
Neutrophils	(2)
Eosinophils (acidophils)	(3)
Basophils	(4)
Agranulocytes	
Lymphocytes	(5)
Monocytes	(6)
Thrombocytes (platelets)	(7)

A brief description of the appearance and function of each is presented here to allow the reader to become familiar with and begin to identify these cells (Fig. 2.3). More detailed descriptions are given in later chapters.

Erythrocytes

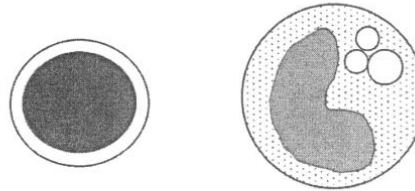
The most common cell encountered in the blood is the **erythrocyte**; overall, there are approximately 1,000 erythrocytes for every leukocyte. On a slide the erythrocyte of most mammals is a round, homogenous, nonnucleated cell that stains pink to salmon to red. In the dog, the center of the cell is thinner than the edges (biconcave) and more lightly stained, but this shape is less pronounced in the other species. The erythrocyte in the members of the camel family (camels, llamas, etc.) is routinely oval, and in birds, reptiles, amphibians, and fish, it is oval and nucleated. The major function of the erythrocyte is to transport oxygen from the lungs to the cells and tissues throughout the body. The carbon dioxide that is generated by the cells is then carried back to the lungs.

Red Blood Cells (Erythrocytes)**White Blood Cells (Leukocytes)****Granulocytes**

Neutrophil

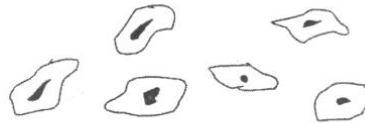
Eosinophil

Basophil

Agranulocytes

Lymphocyte

Monocyte

Platelets (Thrombocytes)**Fig. 2.3.** The seven "formed elements" (cells and cell fragments) of the blood.**Leukocytes**

Scattered among the erythrocytes on the slide preparation are nucleated cells of varying sizes, some of which contain granules that stain various colors. These are the **leukocytes**, of which there are five types. The neutrophil, eosinophil, and basophil routinely have granules present in the cytoplasm (cellular fluid) and are categorized as "granulocytes," while the lymphocyte and monocyte are "agranulocytes." Although the leukocytes have somewhat different individual functions, which are discussed in Chapter 7, in gen-

eral their activities are related to recognizing and responding to any substance foreign to the body, especially potential disease-causing agents such as bacteria, viruses, and fungi.

The most common of the leukocytes in most animals, and the second most common in the rest, is the **neutrophil**. Even with slight differences in appearance between species, it is usually the most easily recognized leukocyte. The neutrophil is somewhat larger than an erythrocyte and is characterized by its densely stained, clumped-appearing nucleus, which is elongate and usually deeply indented or constricted. This clumping often makes the nucleus appear to have two to five separate lobes or segments, which leads to the common names of “segmenter” or “polymorphonuclear” (PMN), meaning the nucleus can take many shapes. When stained, the cytoplasm is clear to pale blue and contains scattered to numerous neutrophilic (gray to light pink) granules. In some species the granules are quite obvious, but in many species they appear as a fine dust or are too small to see clearly with the light microscope, and so the cytoplasm will appear clear. If the nucleus is more ribbon shaped, with parallel sides, it is called a band or stab neutrophil, and if more than five segments are seen, it is referred to as hypersegmented. Differentiating between these developmental stages is an important diagnostic aid and is discussed in Chapter 7.

The **eosinophil** (or acidophil) is only slightly larger than the neutrophil and has a nucleus that may be band shaped or constricted into a bilobed or trilobed appearance. The cytoplasm is usually a light blue but may be difficult to see due to the presence of distinctive granules. The size, shape, number, and staining qualities of these granules vary greatly between species and are so characteristic that the eosinophil can often be used to determine the species from which the sample was obtained. These cytoplasmic granules are called eosinophilic because they chemically attract eosin, the red dye used in staining. In general, they appear pink to orange to salmon colored, and occasionally bright red, in routine laboratory stains. They often refract (bend and scatter) light as it passes through the granules, which makes focusing directly on them difficult, and they may look like bright dots. Since overall staining qualities will vary with different techniques and species, a useful comparison is that the color of the granules should be similar to, or slightly lighter staining than, the surrounding erythrocytes.

The **basophil** is rarely encountered in normal blood smear preparations. Approximately the same size as the neutrophil and eosinophil, it has an elongate to slightly indented nucleus that has less densely staining chromatin. The nuclear outline is often obscured by basophilic granules in the cytoplasm, staining from light or dark purple to almost black. The number of granules may vary from few to densely packed and may be very small and light staining (as in the cat) or larger and very dark (as in the ruminant).

The **lymphocyte** is the most common leukocyte seen in many ruminants and rodents. It is unique among the leukocytes in that when it is stimulated it has the ability to change its size and shape into large, medium, and small forms and it can form more oval cells called plasma cells. The mature lymphocyte is the smallest of the leukocytes, often barely larger than the erythrocyte, and has a round nucleus surrounded by scanty, blue-staining cytoplasm, which is often barely detectable. The nuclear chromatin is coarse and clumped,

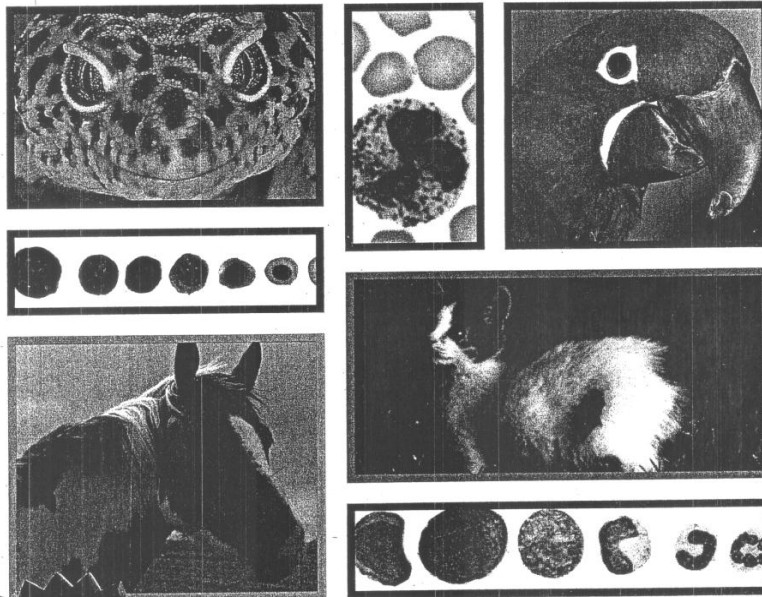
and a small indentation may be present at the nuclear periphery. Much larger lymphocytes are also seen, especially in cattle, with more abundant cytoplasm and a larger nucleus that may become more oblong or rectangular. These cells are easily distorted by surrounding cells. When the cytoplasm stains a more intense blue, the term “immunocyte” is often applied. Some lymphocytes will form plasma cells (plasmacytes), which are oval and have a round, eccentrically placed nucleus in which the clumped chromatin often has a cartwheel appearance. These cells are discussed in more detail in Chapters 7 and 8.

The **monocyte** is the largest of the leukocytes and is similar in appearance in all common species. It is characterized by a pleomorphic, or ameboid, nucleus that can assume various shapes from elongate to rounded and is often in a kidney bean, horseshoe, butterfly, or H shape. The nuclear chromatin is fine, lacy, and smooth, with very little clumping, and therefore stains less densely than the other leukocytes (a characteristic often useful in identification). There is abundant, bluish-gray cytoplasm, which often has a foamy or ground glass appearance and usually contains small to large vacuoles (holes or bubbles). Since the major activity of the monocyte is phagocytosis (the engulfing and digesting of particles), variable granules or particles, and sometimes other cells, may be found in the cytoplasm.

Thrombocytes

The final “formed element” seen in the blood smear is the platelet, or **thrombocyte**. Thrombocytes are not even complete cells but are simply pieces of the cytoplasm of a large cell found in the bone marrow (the megakaryocyte—see Chap. 13) and vary widely in size and shape. Occasionally they may be nearly as large as an erythrocyte (especially in the cat) but more commonly are small and lightly stained and may contain granules. They are usually scattered randomly throughout the smear but will occasionally be seen in small to large clumps that may also include other leukocytes, especially when slow or otherwise improper collection techniques were used in obtaining the sample. This clumping is due to the platelet’s essential function in blood coagulation.

VETERINARY HEMATOLOGY AND CLINICAL CHEMISTRY



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CHAPTER**2**

**SAMPLE COLLECTION,
PROCESSING, AND ANALYSIS
OF LABORATORY
SERVICE OPTIONS**

In the previous chapter, laboratory technology was reviewed. To take advantage of this technology and its medical diagnostic capability, however, samples for the respective procedures must be properly collected and prepared. From this rather vast array of diagnostic options, the veterinarian must also make decisions regarding implementation of these procedures, which will be influenced by several factors. The important factors include the type of practice (e.g., general, outpatient clinic, emergency facility, specialty referral center), geographic location, and practice style of the individuals involved. This chapter presents rules for proper sample processing and guidelines for selecting the appropriate laboratory diagnostic options.

**SAMPLE COLLECTION
AND PROCESSING**

Regardless of the technique or laboratory used for any diagnostic test, obtaining reliable results starts with

proper collection and handling of the sample. Sample collection, processing, testing, and interpretation all must be properly performed as a complete, sequential chain of events for a diagnostic result to have its intended value. For example, even the most reliable test, performed in the most reliable facility and interpreted by the most skilled diagnostician, cannot overcome the error introduced by an inappropriate technique used in sample collection or handling. This section provides guidelines for sample collection and handling that will ensure the initial sequence of events are properly performed.

Containers for Sample Collection

A variety of commercially available tubes are used for blood collection. These tubes contain the appropriate anticoagulant for the various diagnostic procedures and a vacuum for drawing in the appropriate volume of blood. These tubes are commonly known as vacutainer tubes (after the trademark of Becton-Dickinson). The

following commonly used vacuum tubes are described in the approximate order of their frequency of use. Tubes are commonly referred to by their stopper color, which is used to identify the type of anticoagulation system the tube contains (Fig. 2.1).

Red-Top or Serum Collection Tube

The red-top of serum collection tube contains no anticoagulant. Blood that is placed in this tube is expected to clot so that serum may be harvested. This tube is used to collect serum for common biochemical determinations, such as those tests used in creating biochemical profiles.

Lavender-Top Tube

The lavender-top tube contains the anticoagulant ethylenediaminetetraacetic acid (EDTA) salt. This tube is used to collect blood for hematologic determinations. The EDTA anticoagulant results in the most consistent preservation of cell volume and morphologic features on stained films. The liquid tripotassium salt is the most commonly used form of EDTA, and this form is preferred for use in preservation of cell volumes as measured on automated hematology analyzers. Powdered forms are not recommended because of slower, inconsistent mixing with blood that is added to the tube.

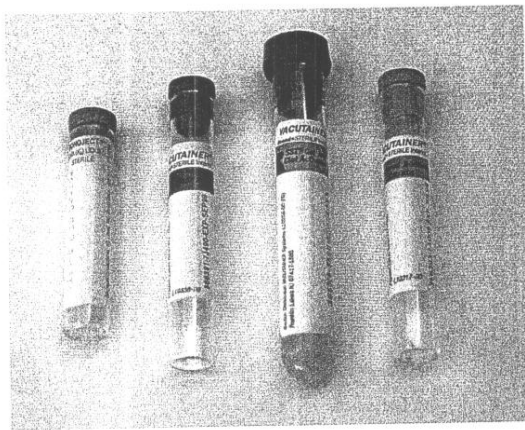


Figure 2.1 Representative vacuum tubes used in the collection of blood samples for diagnostic tests. The tubes are, from left to right, a lavender-top EDTA tube, a red-top tube without anticoagulant, a serum-separation tube, and a blue-top citrate tube. Note that the serum-separation tube contains a yellow gel at the bottom.

Green-Top or Heparin Tube

The green-top tube contains heparin. This anticoagulant is used for certain special biochemistry tests, particularly those that require a whole-blood aliquot for determination and that might be influenced by the presence of other chemical anticoagulation systems.

Blue-Top or Citrate Tube

The blue-top tube contains sodium citrate. It is used for coagulation biochemistry determinations.

Sure-Sep Tube

The Sure-Sep tube is a variation of the red-top tube containing no anticoagulant. The stopper is red with black mottling, and the tube contains a gel that separates packed cell fractions from serum when it undergoes centrifugation. It is convenient for use in situations when centrifugation at the site of collection and transport to the laboratory without the transfer of serum to a separate tube are desirable. The gel physically separates cells from the serum fluid, thus preventing analyte metabolism from occurring at the cell/fluid interface.

Gray-Top or Fluoride Tube

The gray-top tube contains sodium fluoride. Fluoride is not an anticoagulant, however. Rather, it inhibits enzymes in the glycolytic pathway and prevents erythrocytes from metabolizing glucose while whole blood is transported to the laboratory. It is not commonly used.

Tips for Filling Vacuum Tubes

A few simple habits must be developed for appropriately filling tubes:

1. The ratio of blood to anticoagulant volume is designed to be fixed. This is particularly important for hematology and blood coagulation biochemistry tests; therefore, a tube with anticoagulant should be filled to the volume specified for that tube. The amount of vacuum in the tube facilitates this, but the user should watch to ensure that this consistently occurs.
2. When collecting blood for several diagnostic procedures, fill the tube(s) containing anticoagulant first and the tube containing no anticoagulant last. The most commonly used combination of tubes is an EDTA and clot/serum tube. The EDTA tube should be filled first so that any clot formation is minimized. This is unimportant in the tube without anticoagulant, however, because the blood is expected to clot in that tube.

3. Vacuum tubes should be filled using minimal positive force, because forceful passage of blood through the needle may cause hemolysis, which in turn may cause an error in the biochemical measurements. Smaller-gauge needles are more likely to cause hemolysis. An 18- to 20-G needle is best for most collection procedures.
4. Clean venipunctures with no tissue contamination are important. Tissue contamination may result in unwanted platelet aggregation and clotting in samples collected using anticoagulants. As a result, select venipuncture sites (e.g., the jugular vein) that likely will yield the appropriate volume of blood needed for the diagnostic tests being ordered for a given patient.
5. Select a venipuncture site that will yield the desired amount of blood easily. This means being able to draw the blood with little or no collapse of the vein so that blood may be transferred to the anticoagulant tubes as rapidly as possible. Recommended venipuncture sites for diagnostic screening procedures such as a hemogram and biochemical profile include: the jugular vein for small dogs, cats, horses, and cows; and the cephalic or jugular vein in medium to large dogs. These procedures generally require 5 to 12 mL of blood depending on the laboratory and the complexity of the screening procedures.

General Sample Handling Procedures

Hematologic Procedures

Blood collected for a complete blood count (CBC) should be analyzed within 1 hour or be prepared in the proper way for analysis at a later time. If the blood is not analyzed within 1 hour, a blood film should be prepared and the tube refrigerated. Morphologic features of cells may deteriorate rapidly on storage of blood in an EDTA tube; an air-dried blood film preserves the morphology of such cells for later examination. Refrigeration of the blood tube also helps to preserve the cell components that are measured by automated cell-counting systems. For example, cell swelling that could produce artifactual increases in mean cell volume (MCV) and hematocrit occurs as blood is stored in a tube at room or higher temperature. For some analytical systems with differential capability, it is recommended by the laboratory that blood be held at room temperature. Blood should never be frozen, however, because this will result in lysis of the cells. In addition, blood films should not be refrigerated, because water condensation on the glass may damage the cellular morphology.

For hematologic measurements, the EDTA tube should be filled to the specified volume, and tissue contamination during venipuncture should be avoided. Underfilling

the EDTA tube results in excess EDTA, which osmotically shrinks erythrocytes. In turn, this results in falsely decreased packed cell volume and MCV when the microhematocrit procedure is used. Tissue contamination during venipuncture results in platelet aggregation (Fig. 2.2), and this artifactually decreases the platelet concentration as determined by cell-counting systems and may contribute to fluidic obstruction on some hematology instruments.

Clinical Biochemistry Procedures

Blood collected in the red-top tube is allowed to clot for 15 to 30 minutes and then centrifuged to separate the cellular components from the resultant serum. The fluid phase of the blood should be separated from the cellular elements, because cells metabolize certain chemical components in the serum. The most notable example is glucose. If left in contact with cellular elements, glucose is metabolized at a rate of approximately 10% per hour. After centrifugation, serum is harvested by a transfer pipette to a second tube or is dispensed directly to devices for biochemical determinations (Fig. 2.3). Harvested serum should be analyzed quickly; otherwise, it can be refrigerated for as long as 24 to 48 hours. If serum is to be held for longer than 24 to 48 hours, it should be frozen, and serum that is to be held frozen indefinitely (e.g., for archival purposes) should be stored at -70° . Most chemical constituents are stable under these conditions. If serum is frozen and then thawed for analysis, the thawed aliquot should be thoroughly mixed before testing.

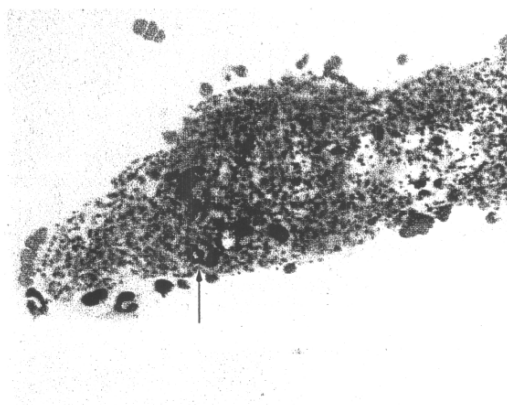


Figure 2.2 Platelet aggregation observed on a stained blood film. Tissue contamination may result in microclots that consist of hundreds of platelets, which falsely decrease the platelet concentration. Microclots also may trap leukocytes. Note the representative leukocyte (arrow); low magnification.

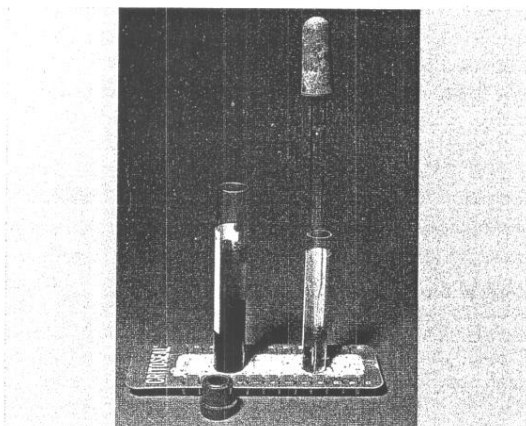


Figure 2.3 Serum preparation for biochemical tests. The tube on the left was allowed to clot and then centrifuged to pack the cells below the serum layer. A transfer pipette is used to transfer serum from the centrifuged sample to the tube on the right.

Serum enzymes require separate consideration regarding storage. A general rule is that for best reliability, serum enzyme activities should be determined within 24 hours of collection. Long-term archival storage of samples for determination of serum enzyme activity is not advised. Data on the exact stability of serum enzyme activity under various storage conditions is difficult to interpret. Knowledge regarding this subject has not been updated in any systematic way during recent years, and historical data were not collected in any consistent manner. Thus, our current understanding of enzyme stability during storage may be summarized as follows: Commonly measured enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase, and alkaline phosphatase, and amylase activities are satisfactorily stable (>70% activity) when stored at 4°C. Freezing, however, may result in considerably accelerated loss of ALT activity. Creatine kinase activity should be measured as soon as possible, because considerable activity is lost after 24 hours regardless of the storage conditions.

Special Procedures

Special laboratory diagnostic procedures are usually performed by centralized or commercial laboratories because of the complexity or specialized instrumentation involved. These procedures are performed less frequently, and they are more dependent on unique requirements of the technology employed by the laboratory undertaking the procedure. As a result, the laboratory

protocol materials for sample collection and handling should be consulted rather than committing these requirements to memory.

ANALYSIS OF DIAGNOSTIC SERVICE IMPLEMENTATION OPTIONS

The veterinarian has several options for obtaining laboratory diagnostic data. These may be generally considered as falling into three categories:

1. In-house (performed on the premises).
2. Commercial veterinary laboratory.
3. Human laboratory or community hospital.

Several factors should be considered when formulating a strategy for using one (or more) of these options. The veterinary facility should self-assess the following:

1. Type of practice (e.g., general practice, outpatient clinic, emergency facility, specialty referral center).
2. Geographic location (proximity to reliable service options).
3. Practice style of the individuals involved.
4. Willingness to implement and evaluate quality-assurance programs.
5. Willingness to invest the time to evaluate and troubleshoot diagnostics with varying degrees of complexity.
6. Willingness to invest in a good microscope and training of personnel regarding basic clinical microscopy.
7. Desired turnaround times.
8. Ability to invest in instrumentation and training for the operators.

Advantages and Disadvantages of In-House Laboratory Testing

Advantages of in-house laboratory testing include rapid turnaround time and control over when testing is performed relative to when samples are drawn in a particular practice setting. In-house testing may also have economic advantages in certain situations.

Disadvantages of in-house laboratory testing include the issue of technical operator expertise for basic laboratory technology, which may not be available or affordable in many veterinary facilities. Attention to detail and quality assurance also must be managed by someone on site, and an investment in equipment is required. In addition, access to a clinical pathologist to help with the characterization of abnormal screening tests, particularly blood film analysis for hematology, must be cultivated,

and arrangements for specialized testing to supplement in-clinic diagnostic tests must be procured.

Advantages and Disadvantages of Commercial Veterinary Laboratories

The major advantages of commercial veterinary laboratories are the cost leveraging of automated instrumentation and centralized testing volume, a complete menu of testing services, professional oversight of technical performance, and pathology support. Because the automated instrumentation is dedicated to animal-specific diagnostics, it is usually already adapted for the proper analysis of animal samples. Quality-control programs are usually implemented as well, but these are variable.

The major disadvantages of commercial veterinary laboratories include relatively fixed turnaround times, which are dictated by sample transportation logistics. In addition, sample transportation is a major part of the cost of the service. Pathology support and consultation may be variable as well.

Advantages and Disadvantages of Human Laboratory Facilities

The advantage of human laboratory facilities is that they may be the only available option in less-populated areas. The disadvantages, however, are considerable. The instrumentation, particularly for hematology, is usually not modified for animal-specific diagnostics, and knowledge about the consequences is often lacking. Animal-specific pathology support is usually nonexistent or minimal. The technologists do not have training in veterinary hematology, and nobody on site can provide that training. In addition, turnaround times for animal testing may not receive the appropriate priority relative to the primary purpose of the laboratory.

FACTORS TO CONSIDER WHEN COMMITTING TO IN-HOUSE TESTING

Investment in Instrumentation

Acquiring a diagnostic capability in chemistry and hematology requires an investment of approximately \$10,000 to \$25,000—or more. The cost of equipment has somewhat stabilized in this range, but the technical capability for this investment continues to improve. For example, a diagnostic capability in hematology that cost in excess of \$80,000 during the 1980s may now be obtained for \$10,000 to \$15,000. The useful technical life span of most instrumentation should be viewed as being from 5

to 7 years. Lease plans may facilitate the acquisition of instrumentation in ways that involve planned replacement at 3- to 7-year intervals.

Commitment to Personnel

Commitment to personnel requires hiring—and retaining—a technologist who is capable of reliable performance in diagnostics. Essential elements include an understanding of the basic laboratory technology, an ability to perform these procedures, a willingness to implement quality control, and a mindset that allows the technologist to seek consultation when he or she is confronted with uncertainty.

Commitment to Quality Assurance

A commitment to quality assurance involves a willingness to invest in periodic training regarding diagnostic technology for the personnel who perform these procedures as well as in the oversight of a regular quality assurance program. The latter involves regular monitoring of equipment accuracy and precision using commercial control materials with known target values. This may cost from \$100 to \$300 per month for materials.

Establishing a Pathology Consultation Relationship

A working relationship with a veterinary clinical pathologist to provide help with data interpretations and morphologic assessments in difficult cases, as well as cytopathology support, is highly desirable. A relationship with an anatomic pathologist is also required for interpretations of surgical biopsy specimens.

The Business Plan

Veterinarians who are considering in-house testing must have a mind-set that allows them to use diagnostics liberally as part of their practice style. Instrument salespersons may make a compelling case for how one or two CBCs per day will pay for the cost of an instrument system. The same occurs in chemistry as well. First and foremost, these schemes are profitable for the seller, but this may or may not be true for the buyer. One should not make this investment without first analyzing the costs of various alternatives, such as the use of external laboratories. Veterinarians who perform only occasional diagnostic workups likely are better off using an external laboratory. Alternatively, diagnostics may be viewed as a source of revenue if the practice style calls for a combination of frequent diagnostic workups, preanesthetic testing, and wellness testing programs. Thus, a business

plan should be created that projects the number of diagnostic tests to be performed across the practice caseload. Multiplying these numbers by the projected internal charge for laboratory tests will yield the gross revenue of the proposed in-house testing effort. Recommended target values are the charges for similar tests imposed by a veterinary commercial laboratory in the region. The projected gross revenue then should be compared with the projected costs, including instrumentation amortization, consumable supplies, personnel, training, quality assurance, and time for supervision.

For chemistry, one must also consider the style of use. Most of the currently available systems are not economically favorable for performing biochemical profiles in-house. For example, the cost of consumables per test with an in-house system may easily exceed \$1 to \$3 per test, whereas a complete biochemical profile may be obtained from a laboratory for approximately \$16. With these circumstances, it makes sense to use in-house chemistry testing for single tests or mini-panels, but not for profiling (unless other laboratory options are not available).

FACTORS TO CONSIDER WHEN SELECTING EXTERNAL LABORATORY SERVICES

Instrument Adaptation

Instrumentation must be suitably adjusted for animal blood testing. This is particularly important regarding hematologic analyses. Such adaptation is likely to occur in veterinary commercial laboratories, and it is much less likely to be found in human hospital laboratories that analyze animal samples as a secondary priority.

Sample Pick-Up Service

Many veterinary laboratories offer once or twice daily sample pick-up service to facilitate the shortest possible time from sample collection to the return of results. The trade-off is that courier services represent a considerable fraction of the cost of the laboratory service. Human laboratory facilities usually rely on users to transport samples to the facility.

Appropriate Turnaround Time

In general, the rate-limiting step is transporting the sample to the laboratory. The trend toward consolidation of laboratory services, however, often results in very large transportation distances, thus extending the turnaround time. Once a sample arrives at the laboratory, most facilities perform the analyses as rapidly as possible and then return the results by fax or electronic transmission. Laboratories that prioritize animal samples behind a busy human diagnostics schedule may not provide convenient timing for the delivery of results.

Species-Specific Ability

The laboratory should have the ability to recognize and interpret species-specific morphologic and pathologic abnormalities. In addition, the laboratory should be able to provide knowledgeable evaluation of abnormalities in data and morphology on blood films and cytology.

Telephone Consultation

The veterinary user must be able to consult with laboratory staff and pathologists regarding abnormal or unusual data generated by the laboratory.

Decision Process

The analysis of one's diagnostic options may be summarized as follows: The decision process for implementing diagnostic support is complex, and this complexity is enhanced by rapidly changing technologies and services. It is advisable to run some experiments to facilitate this analysis. To maintain flexibility when uncertainty exists, it is advisable to avoid entering long-term purchase or service agreements.

SUGGESTED READINGS

- Jain NC, ed: Schalm's veterinary hematology. 4th ed. Philadelphia: Lea & Febiger, 1986.
- Kaneko JJ, Harvey JH, Bruss ML, eds: Clinical biochemistry of domestic animals. 5th ed. New York: Academic Press, 1997.



The Clinical Chemistry - Point of Care

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2. Preanalytical control

2.4. Blood Testing – Choosing The Right Specimen at The Point of Care

2.4.1. Whole blood, plasma or serum?

Testing is generally performed on one of three different specimens: whole blood, plasma, or serum and there are numerous criteria that determine which specimen is most suitable for a particular test and setting.

Whole blood

Whole blood is a venous, arterial or capillary blood sample in which the concentrations and properties of cellular and extra-cellular constituents remain relatively unaltered when compared with their in-vivo state. Anticoagulation in-vitro stabilizes the constituents in a whole blood sample for a certain period of time.

Diagnostic manufacturers have developed small whole blood analyzers as a near-patient alternative to the conventional plasma method. Most POCT uses whole blood.

Serum or plasma

The choice between serum and plasma for centralized chemistry testing is an area of great interest in terms of specimen selection. Early, serum has been the conventional standard for most chemistry test.

Today, the choice between serum and plasma is an large part a decision based on the unique requirement and priorities of individual lab. When the priority is TAT (turnaround time), plasma has a clear advantage since it can be centrifuged immediately upon collection. However, in settings where the mean time between phlebotomy and specimen arrival at the laboratory exceeds recommended serum specimen clot times, or where the laboratory does not otherwise hold specimens, the advantages in TAT with plasma are not realized. Labs may also find that serum samples lead to more instrument downtime than plasma samples as a result of fibrin formation.

This claim needs to be verified by each laboratory, since specimen performance is highly dependent on adherence to recommended handling conditions for both serum and plasma tubes.

Plasma

Plasma is the virtually cell-free supernatant of blood containing anticoagulant obtained after centrifugation.

Centrifuge the anticoagulated blood (citrate, EDTA or heparinized blood) for at least 15 minutes at 2000 to 3000 g to obtain cell-free plasma.

Advantages of using plasma

The following aspects support the preferential use of plasma versus serum in laboratory medicine:

- **Time saving:**

Plasma samples can be centrifuged directly after sample collection, unlike serum, in which coagulation is completed after 30 minutes,

- **Higher yield:**

15 to 20 % more in volume of plasma than of serum can be isolated from the same volume of blood.

- **Prevention of coagulation-induced interferences:**

Coagulation in primary and secondary tubes that were already centrifuged, may block suction needles of the analyzers when serum tubes are used; this can be prevented by using anticoagulants.

- **Prevention of coagulation-induced changes:**

The coagulation process changes the concentrations of numerous constituents of the extra-cellular fluid beyond the maximum allowable limit (8, 15). The changes are induced by the following mechanisms:

- a. Increase in the concentrations of platelet components in serum as compared to plasma (e.g. potassium, phosphate, magnesium, aspartate aminotransferase, lactate dehydrogenase, serotonin, neurone-specific enolase, zinc). Release of amide-NH₃ from fibrinogen induced by action of clotting factor XIII.
- b. Decrease in the concentration of constituents in serum as a result of cellular metabolism and the coagulation process (glucose, total protein, platelets).
- c. Activation of the cell lysis of erythrocytes and leukocytes in non-coagulated blood (cell-free haemoglobin, cytokines, receptors).

Certain constituents should only be measured in plasma (e.g. neurone-specific enolase, serotonin, ammonia) to obtain clinically relevant results.

Disadvantages of plasma over serum

The addition of anticoagulants can interfere with certain analytical methods or change the concentration of the constituents to be measured:

- a. Contamination with cations: NH₄⁺, Li⁺, Na⁺, K⁺.

Assay interference caused by metals complexing with EDTA and citrate (e.g. inhibition of alkaline phosphatase activity by zinc binding, inhibition of metallo-proteinases, inhibition of metal-dependent cell activation in function tests, binding of calcium (ionized) to heparin (16)).

- b. Interference by fibrinogen in heterogeneous immunoassays (15).
- c. Inhibition of metabolic or catalytic reactions by heparin: e.g., Taq polymerase in the polymerase chain reaction (PCR) (17)
- d. Interference in the distribution of ions between the intracellular and extracellular space (e.g. Cl^- , NH_4^+) by EDTA, citrate (8).
- e. Serum electrophoresis can be performed only after pre-treatment to induce coagulation in plasma.

Serum

Serum is the undiluted, extracellular portion of blood after adequate coagulation is complete.
When plasma coagulation is complete, the sample should be centrifuged for at least 10 minutes at a minimum speed of 1500 g.
When separating serum or plasma, the temperature should not drop below 15 °C or exceed 24 °C.

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EQUINE DISEASE QUARTERLY

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Commentary

KNOWLEDGE IS POWER. Actually, it's much more accurate to say "*Factual knowledge in context is power.*"

Polymerase chain reaction (PCR) testing is an extremely sensitive test for biologic agents in horses. However, a positive PCR for *Salmonella* can indicate either the presence of live bacteria in the sample or dead bacteria with DNA still present. So, is the horse actively shedding live bacteria, or just DNA? The positive PCR test is a fact, but interpretation is everything.

Drug testing of human and animal samples for illegal substances has become extremely sensitive, in some instances detecting one part per billion of a substance. Is every positive drug test indicative of illegal drug use? The answer to this question is of obvious interest to high-caliber human athletes as well as horse owners.

Interpretation of ultra-sensitive test results has its own challenges. With the rapid advances in diagnostic testing technology in veterinary and human medicine, some people assume that test results are 100% sensitive (all positive samples are detected) and 100% specific (all negative samples are detected). However, no such test exists. In current biological diagnostic systems, some false positives and some false negatives are expected, with some procedures having 90+% sensitivity and specificity.

Even DNA testing is not 100% accurate, as the likelihood of confirming identity or paternity is dependent upon the number of DNA markers used in the test. The more markers tested, the greater the probability that two samples will match. However, probability is never 100%. Only an exclusion of identity or paternity can be made with 100% accuracy.

This doesn't mean that all diagnostic testing is worthless because of less than 100% sensitivity. Understanding of current technology—which is incredible compared to diagnostic capability of only

10 years ago and continually improving—makes diagnostic interpretation possible, and multiple tests can confirm results.

Weather forecasting is not an exact science, but horse owners have a significant interest in weather conditions. If property or animals are lost due to adverse weather events, people are affected both financially and emotionally. In addition, both fire and drought can directly affect nutrition, water supplies, and feed costs. Weather and drought predictions are based on history, facts, advanced computer modeling, and interpretation—not a crystal ball. Many people have complained about weather forecasts, but modern weather predictions for significant storms are impressive considering the difficulty of anticipating Mother Nature's tricks.

In today's world of high-speed communications, some misinformation or unverified information—even rumors—can run wild. The test of accuracy is in the reliability of the information source, understanding the overall situation, and monitoring updates for changes and corrections.

Whether it's accurate interpretation of diagnostic test results or predictions of adverse weather conditions, *Factual information in context* is power!

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First Quarter 2007

The International Collating Centre, Newmarket, England, and other sources reported the following disease outbreaks:

Contagious Equine Metritis was confirmed in one non-Thoroughbred stallion (Franches-Montagne) at the National Stud in Switzerland. Continuing the 2006 last quarter report concerning isolation of *Taylorella equigenitalis* from imported Lipizzaner stallions in Wisconsin, the USDA confirmed that the stallions, after completing two series of antibiotic treatments, cultured negative for the organism. In addition, each stallion was bred to two test mares, which also tested negative for the organism. The stallions were released from quarantine on March 7, 2007.

Cases of respiratory disease caused by equine herpesvirus (EHV) were widely reported from a variety of horse breeds in France. Horses among the Thoroughbred racing population at Sha Tin racecourse, Hong Kong, displayed fever (ranging from 101° to 103° F), which was confirmed as caused by EHV-1. Over 100 horses were affected during a period of 60 days, representing 12% of the racing population. EHV-1 was diagnosed as the cause of death in a donkey that died in the United Kingdom with severe tracheitis.

Abortions attributable to EHV-1 were reported among Standardbred mares in France. Ten cases were reported from Ireland, 14 cases on six premises from Japan, eight cases in the United Kingdom, and 14 cases in Central Kentucky, six of which occurred among vaccinated mares on one farm.

Cases of the paralytic form of EHV-1 were identified in one Thoroughbred mare reported from Japan and a Welsh Cob in the United Kingdom. Several outbreaks were reported in the United States, all

characterized by low morbidity but high mortality. In January, cases were reported on two premises in Connecticut, in February in Wisconsin with two fatalities, and in New York with one fatality. Also during February, six cases with three fatalities occurred at the Marion duPont Scott Equine Medical Center, Leesburg, Virginia. The facility was closed under a state quarantine but reopened to accept equine patients at the end of March. During March, cases were diagnosed on one premise in Florida with two fatalities; in California, six cases were diagnosed with three fatalities on two premises; and in Maine, with one fatality.

A single case of coital exanthema (EHV-3) was diagnosed in a Thoroughbred stallion in the United Kingdom.

All restrictions on premises with respect to the outbreak of equine infectious anemia (EIA) in Ireland were lifted on March 21. In Italy extensive testing of the equine population for EIA is under way, with 29 positives identified during the first quarter.

Equine influenza was reported among a variety of breeds in France, and an extensive outbreak was confirmed among unvaccinated Standardbreds in training stables in Sweden. An unvaccinated Warmblood horse recently imported from Holland was confirmed as positive for influenza in the United Kingdom.

Strangles was widely reported in Ireland, and cases were reported on three premises in Switzerland among non-Thoroughbred horses.

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Wildfires, Droughts, & Lightning

Wildfires in the United States in the first six months of 2007 have been significant, with hundreds of thousands of acres affected in Georgia, Florida, New Jersey, Minnesota, and even Catalina Island, California. The National Interagency Wildfire Center has predicted the hot zones of wildfire risk through August (http://www.nifc.gov/nicc/predictive/outlooks/season_outlook.pdf).

While wildfires are common natural disasters in the western United States, the wildfire forecast includes all of Florida and the southern states of Mississippi, Alabama, Georgia, and South Carolina. Additionally, western North Carolina and Virginia and portions of Alaska are at high risk.

One obvious factor is the drought conditions in many of these states. Alarming, the U.S. Drought Monitor (at <http://www.drought.unl.edu/dm/monitor.html>) showed abnormally dry conditions in wide areas of the United States as of June 1—well before the tinder dryness of later summer months.

Multiple causes for wildfires exist, some due to human factors including campfires, burning brush, smoking, fireworks, use of flares or power tools (welding, grinding, and other power tools), and outright arson. Other hazards include downed power lines, spontaneous combustion of hay and bedding, and electrical fences in contact with dried vegetation.

A natural cause for fires is lightning. The United States has more than 25 million lightning flashes per year, according to the National Weather Service. Each lightning spark can reach more than five miles, and lightning strikes can pack 50,000° F temperatures and 100 million electrical volts.

From 2000-2007, the University of Kentucky Livestock Disease Diagnostic Center (LDDC) diagnosed 101 cases of lightning strike in horses. While 88 (87%) of these cases occurred in the

summer months (May through August), cases also occurred in February (1), April (4), September (7) and October (1). These figures represent only those horses brought to the LDDC for necropsy, but they suggest the monthly incidence of the lightning-associated deaths for one geographic area, since most Central Kentucky's equine necropsies are performed at the LDDC.

Droughts and lightning increase the risk of wildfires, all which have a profound impact on horses and farms. An evacuation plan is a must no matter where horse farms are, since barn fires and wildfires can occur anywhere under the right conditions.

All wildfires produce noise, heat, and smoke, which trigger panicked behavior in horses, making them challenging to handle, let alone load into a trailer. Early evacuation is therefore essential via preplanned routes and preparation.

Suggestions for horse farm owners to minimize risks are to clear back brush and low vegetation at least 30 feet from all buildings and clear leaves, branches, and other flammable materials from roofs. Trees and brush, once ignited, are more difficult to extinguish than dried grasses. Coniferous trees and some brush contain a substance called sclerophyll, which can be explosive when heated. Also, some tall ornamental grasses are extremely flammable when dry and should also be planted well away from any structure.

Continued awareness of drought conditions in the region and the status of wildfires can help in decision making.

Preparation for all types of disasters (including wildfires) involving livestock is well documented in the free online course "Livestock in Disasters" offered by the Federal Emergency Management Agency (<http://training.fema.gov/IS/>). General preparation guidelines for homes are available at <http://www.fema.gov/>.

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✱ BenZoylEcgonine Thresholds in Horse Urine

In 1985, the Kentucky Racing Commission directed the Equine Pharmacology program at the University of Kentucky to work on improving testing for performance-enhancing drugs. The outcome was the introduction of Enzyme-Linked-ImmunoSorbant Assay (ELISA) testing into racing. ELISA tests are exceptionally sensitive, detecting drug/drug metabolites at low parts per billion (nanograms/ml, equivalent to one second in 32 years) or high parts per trillion (picograms/ml, or one second in 32,000 years) concentrations. This high sensitivity is essential for the detection of illicit drug use in horses. However, the regulatory authorities very soon ran into the problem of these tests detecting minuscule traces of BenZoylEcgonine [BZE] in horse urine.

This is a problem because BZE is the major urinary metabolite of cocaine, which is very efficiently excreted in horse urine. Exposure to 1 mg of cocaine can yield 100 ng/ml (100 ppb) of BZE in horse

urine. Since a BZE ELISA can detect 1 ng/ml or less of BZE, these tests can theoretically detect exposure of a horse to one hundredth of a milligram of cocaine.

To put this into perspective, about 300 metric tons of cocaine are imported into the United States each year, and paper currency is "highly" contaminated with cocaine. In one study on 136 one-dollar bills, 79% carried detectable cocaine, 50% carried microgram amounts, and one bill carried 1.3 mg. If 1.3 mg were given to a horse, it could easily yield a 100 ng/ml detection, which is far below that required to influence racing performance.

Reviewing these matters, Dr. C. Koliass-Baker (2002) noted that 2.5 mg of cocaine, an amount sufficient to yield detectable urinary concentrations of BZE for 24 hours, "could easily be transferred from a cocaine abuser's hands to the mouth or muzzle of a horse" and yield concentrations similar to those "that are occasionally found in urine samples collected from show and race horses." Similarly, Dr. Scott Waterman of the Racing Medication and Testing Consortium noted that "the presence of cocaine in a horse's blood or urine is not a sure sign that somebody is trying to fix a race, because trace amounts of cocaine could be spread by casual contact with human users" (C. Wilson, *The Associated Press*, 12/8/2005).

When sensitive BZE tests were introduced in California, the outcome was dramatic, and within weeks a number of California trainers, some very prominent, were associated with "trace" BZE identifications. When the dust settled, a number of important administrative changes had occurred.

The first change in California was the creation of an equine medical director position to oversee drug testing and other procedures. The second change was the introduction of thresholds, or cut-offs, for certain environmental substances in racing horses. These changes follow well-established precedents in human medicine. The equine medical director position is equivalent to a human medical review officer, and it is structured to address situations such as the multiple BZE "identifications" in California racing in the late 1980s.

The introduction of urinary cut-offs for BZE were modeled on the 150 ng/ml BZE cut-off present in human workplace drug testing. In 1999, Ohio introduced a BZE cut-off of 150 ng/ml. This threshold has since been adopted in Louisiana, Illinois, and Oklahoma, and lower cut-offs for BZE are in place in Washington state and Florida. And most recently, recognizing the regulatory implications of this problem, the U.S. Racing Medication and Testing Consortium has created an environmental contaminants subcommittee to evaluate and recommend approaches to this problem. It is chaired by Kent Stirling, executive director of the Florida Horsemen's Benevolent and Protective Association.

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Equine Leptospirosis

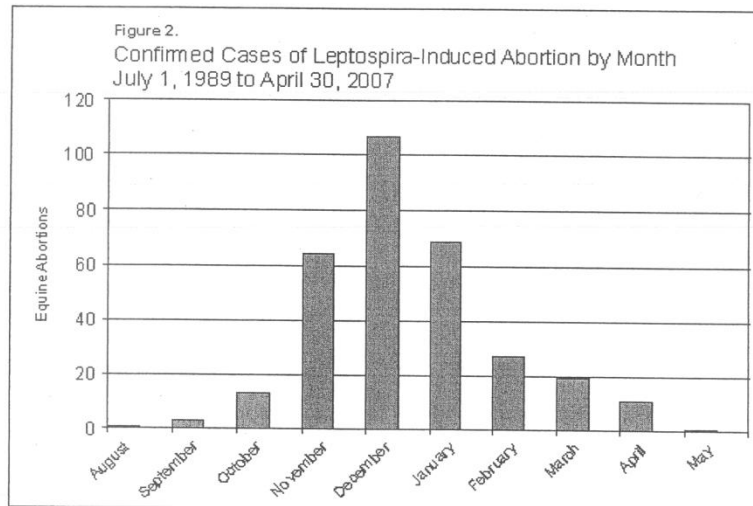
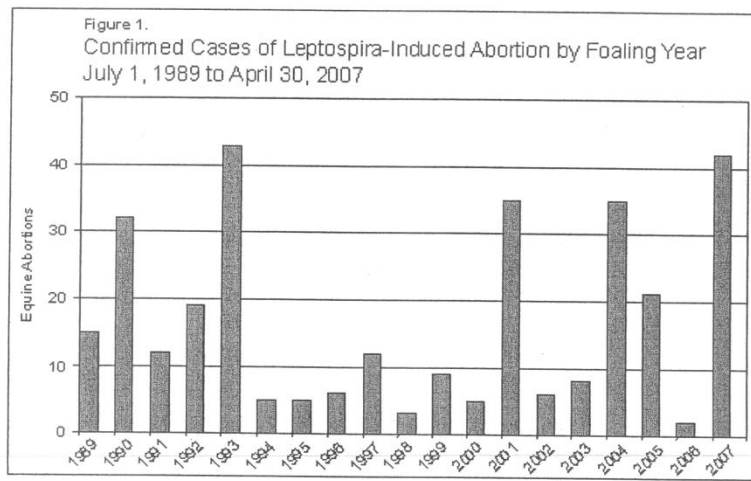
The last report of equine leptospiral-induced abortions was in the April 2004 issue of *Equine Disease Quarterly*. Since this report, additional cases of leptospiral-induced abortions have been diagnosed at the Livestock Disease Diagnostic Center at the University of Kentucky. For reporting purposes, a foaling year begins July 1 and ends June 30 of the subsequent year. Therefore, the following report includes cases diagnosed for the foaling years 2005, 2006, and most of 2007.

Sixty-five leptospiral-induced abortions have been diagnosed during the last three foaling years (July 1, 2005, through April 30, 2007). Breeds of horse and number of cases involved were Thoroughbred, 59; Standardbred, 5; and mixed breed, 1. Over the three-year period, the number of leptospiral-induced abortions on farms varied, with 38 farms having a single leptospiral-induced abortion, two farms having two abortions each, three farms with three abortions each, one farm with four abortions, and a single farm with 10 abortions over the three-year period.

Leptospirosis is a zoonotic bacterial disease with global distribution. Virtually all species of mammals are susceptible. The bacteria, called "spirochetes," are 6-20 microns in length, 0.1-0.2 microns in width, motile, and helically coiled. The genus is divided into multiple species and is further divided into more than 250 serovars. The term "serovar" commonly is used to describe a specific strain of *Leptospira* spp. The serovars with antigens in common are placed in serogroups for diagnostic convenience. The predominant serovar affecting horses varies with country and region. In Central Kentucky, serovars commonly affecting horses and causing infections include *L. interrogans* serogroup Pomona serovar *kennewicki* and *L. kirschneri* serogroup Grippotyphosa serovar *grippotyphosa*. Organisms from serogroup Hardjo rarely have been detected in horses in Central Kentucky.

Serologic results indicated that serovar *kennewicki* of the Pomona serogroup was responsible for 50 (77%) of the abortions and *grippotyphosa* for nine (14%) of the leptospiral-induced abortions for the past three foaling seasons. The serovar was undetermined for six (9%) abortions. Eight (12%) of the leptospiral-induced aborted fetuses were serologically negative. Microscopic agglutination testing and the diagnosis were made via the identification of spirochetes by direct fluorescent antibody test, microscopic identification with the Warthin-Starry staining method, and/or maternal serology.

The Livestock Disease Diagnostic Center has diagnosed 315 cases of leptospiral-induced abortion in Central Kentucky over the past 19 foaling seasons. Almost all cases were due to either *kennewicki* (260 cases, 83%) or *grippotyphosa* (33 cases, 10%). **Figures 1 and 2** detail, by foaling year and month, the number of confirmed cases of leptospiral-induced abortions or neonatal deaths for this period.



Prevention and Control of Leptospirosis

- Since no approved vaccine against leptospirosis in horses is available, prevention of leptospiral-induced abortion is best achieved by minimizing exposure to the bacteria. Prevention is aimed at avoiding direct contact with the urine of wildlife and cattle. Indirect contact with urine that might be present in environmental water sources, feed, and bedding should also be avoided. Practical measures that can be taken to reduce exposure to potentially urine-contaminated areas include strict control of wildlife around barns and feeding hay and grain off the ground. Other domestic species on the premises should be vaccinated against leptospirosis if an approved vaccine exists for that species.
- Following a leptospiral abortion, the area where the mare aborted should be thoroughly cleaned. The aborting mare should be isolated, since shedding of leptospira can continue for weeks. Antibiotic therapy can shorten the period of shedding.

- Serologic testing of pregnant mares can identify mares possibly at risk of aborting due to leptospirosis. High-titer mares should be isolated, and negative, or low-titer, mares should be retested in two to three weeks. A course of antibiotics in mares with high titers may prevent or lessen fetal infection and prevent abortion, although this treatment has not been evaluated in controlled studies.

For additional information concerning leptospirosis in horses see Donahue, J.M. and N.M. Williams: Emergent Causes of Placentitis and Abortion. *Vet Clin North Am, Equine Pract*, 16: 443-455, 2000.

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University of Kentucky, Lexington, Kentucky**

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Rabies in Kentucky

In 2006, the Kentucky Department for Public Health, Division of Laboratory Services (Frankfort) and the Breathitt Veterinary Center (Hopkinsville) received 1,126 animal specimens for rabies testing. Of these, 77 (6.8%) were unsuitable for testing because of decomposition or extreme trauma to the brain. The distribution of rabies-positive animals is shown in **Figure 3**.

This statewide distribution of rabies cases may not be representative of the true incidence of rabies, since detection depends upon submission of proper samples to a testing laboratory. Almost all of the samples received were a result of suspicious behavior of the animal in connection with a human being or domestic animal.

Of the 1,126 animals submitted, 30 proved to be rabies positive. Of these rabid animal cases, 24 involved a bite or physical contact with a human or domestic animal. While skunk is the predominant rabies variant in Kentucky, the raccoon variant responsible for the Mid-Atlantic states' rabies epizootic is present in adjoining West Virginia and Tennessee. Multiple federal and state agencies are actively involved in preventing the spread of raccoon rabies into Kentucky.

2007 Update

As of May 17, 2007, seven cases of animal rabies have been confirmed in six Kentucky counties. The animals included three dogs, two horses, one bat, and one skunk. Human exposure to the rabid animals was involved in four cases (including one horse), with one animal exposure.

Domestic animal rabies cases and human exposures emphasize the need for rabies vaccination. Licensed rabies vaccines are available for horses, dogs, cats, ferrets, cattle, and sheep. Kentucky state law requires that dogs, cats, and ferrets be vaccinated against rabies by 4 months of age.

The human post-exposure rabies vaccination regimen is five doses of vaccine over 28 days plus a rabies immunoglobulin shot; no such treatment is available for unvaccinated, rabies-exposed animals. The human post-exposure series costs approximately \$1,500, not including medical visits and administration. Vaccination of domestic animals, by comparison, is extremely inexpensive.

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Frankfort, Kentucky

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Your Input Is Requested

Help us ensure that we are providing useful information by suggesting topics of specific interest to you.

Go to http://www.ca.uky.edu/gluck/Q_survey07.asp on the Web to list equine health topics about which you would like to read and to make other comments. Then, press "submit." No postage necessary!

Our goal is to provide the most up-to-date, factual information possible to the equine industry.

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To receive a printed copy of the *Equine Disease Quarterly* in the mail, send us your name and **postal** (not e-mail) mailing address. There is no charge.

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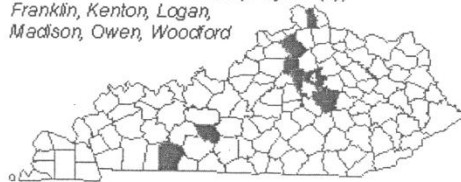
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Figure 3.

Rabies Cases in Kentucky, 2006

*Kentucky Department for Public Health
Division of Epidemiology and Health Planning*

Bats: 11 cases—Edmonson, Fayette (4), Franklin, Kenton, Logan, Madison, Owen, Woodford



Cats: 4 cases—Adair, Butler, Meade, Todd

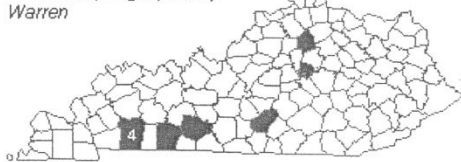


Cows: 1 case—Franklin



Dogs: 5 cases—Barren, Christian, Marion, Scott, Taylor

Skunks: 9 cases—Adair, Christian (4), Jessamine, Logan, Scott, Warren



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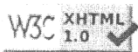
Diane Furry

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Last Updated: Tuesday, October 18, 2011

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Patrice Underwood, Minnesota Horsemen's Benevolent & Protective Association

Jack Walsh, Minnesota Horsemen's Benevolent & Protective Association

Dr. Scot Waterman, Executive Director, Racing Medication and Testing Consortium



**THERAPEUTIC MEDICATIONS ROUTINELY USED AND IDENTIFIED AS NECESSARY BY THE
VETERINARY ADVISORY COMMITTEE:**

First Priority Group

1. Acepromazine
2. Butorphanol
3. Detomidine
- 4. Glycopyrrolate
5. Lidocaine
6. Mepivacaine
- 7. Methocarbamol
- 8. Pyrilamine

Second Priority Group

9. Boldenone
10. Dantrolene
11. Dexamethasone
- 12. Firocoxib
13. Fluphenazine
14. Hydroxyzine
15. Nandrolone
16. Stanozolol
17. Testosterone

Third Priority Group

18. Albuterol
19. Betamethasone
20. Diclofenac
21. Methylprednisolone
22. Reserpine
23. Triamcinolone
24. Trichlormethiazide
25. Xylazine

Fourth Priority Group

26. Atropine

27. Beclomethasone
28. B uscopan
29. Cromolyn
30. Isoxsuprine
31. Pentoxifylline
32. Phenytoin
33. Prednisolone

Fifth Priority Group

34. Diazepam
35. Dipyrrone
36. Fluorprednisolone
37. Guaifenesin
38. Isoflupredone
39. Prednisone

Research Already Underway


40. Aminocaproic Acid
41. Carbazochrome
42. Clenbuterol
43. Procaine Penicillin

Already in body of model rules

44. Cimetidine
- <— 45. DMSO —>
46. Flunixin
47. Furosemide
48. Ketoprofen
49. Omeprazole
49. Phenylbutazone
50. Ranitidine


Medication: Past, Present, and Future in Racing and Performance Horses

Scot Waterman, DVM
Executive Director



Racing Medication & Testing Consortium

- Began in 2001 at a facilitated meeting hosted by the AAEP to assess whether uniform medication rules were achievable
- RMTC Goals: uniform rules, standardized testing, available money for research, improved security, information resource for racing commissions
- Incorporated as a 501 c (3)



Board of Directors

<ul style="list-style-type: none"> ■ AAEP ■ AQHA ■ Arabian Jockey Club ■ California TB Trainers ■ Churchill Downs ■ Del Mar ■ Hambletonian Society ■ Harness Tracks of America ■ The Jockey Club ■ Jockeys' Guild ■ Keeneland ■ KTA 	<ul style="list-style-type: none"> ■ Magna Entertainment ■ National HBPA ■ NTRA ■ NYRA ■ Oak Tree ■ RCI ■ THA ■ TOBA ■ TB Owners of California ■ TRA ■ TRPB ■ USTA
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Racing Medication & Testing Consortium

- Diverse, large Board can be difficult but it ensures that no single agenda can drive the development of a rule, supermajority required for language to pass
- Standard approach is to divide work up into sub-committees and bring the consensus back to the full Board
- Language is either adopted by the Board, edited and then approved or sent back to the sub-committee



Veterinarian involvement in the RMTC

<ul style="list-style-type: none"> ■ Board members <ul style="list-style-type: none"> - Rick Arthur (Oak Tree) - Nancy Goodman (AQHA) - Ted Hill (The Jockey Club) - Bobby Lewis (AAEP) - Gregg Scoggins (MEC) 	<ul style="list-style-type: none"> ■ Ex officio <ul style="list-style-type: none"> - Glenn Blodgett (AQHA) - Ron Jensen (Advisor) - Milton McLare (AAEP) - Wayne McIlwraith (AAEP) ■ Committees <ul style="list-style-type: none"> - Tomi Brokken - Gary Norwood - Dan Wilson - Tom Tobin - Mike Weber
---	---

Model Rules Phase 1 (2005)

- Prohibited Practices
- Drug Classifications/Categorization
- Permitted Therapeutics:
 - Voluntary use of furosemide with post-race regulatory control
 - One of three NSAIDs - Bute, Banamine or Ketoprofen - at 24 hours
- Adopted now by 32/38 racing jurisdictions



Uniform Penalties (2006)

- Three-tiered grid system simplified existing RCI penalties
- Significantly increases penalties for illegal and prohibited performance-enhancing drugs
- Reduces penalties for therapeutic medication mistakes
- Redefines trainer responsibility
 - Mitigating and aggravating circumstances "checklist" for stewards
 - Added penalties for other culpable licensees: veterinarians, owners and grooms
- Adopted as rule of policy in 25 states



Model Rules Phase 3 (2007)

- Regulation of anabolic steroids
 - Thresholds for four FDA-permitted or endogenous anabolics
 - Stanozolol
 - Boldenone
 - Nandrolone
 - Testosterone
 - 30-45 days withdrawal time
- Out-of-competition testing
 - Blood-doping agents - EPO & Darbipoietin
 - Illegal & prohibited drugs



Model Rules-Present & Future Withdrawal Times Research

- Identify drugs with legitimate uses
- Prioritize drugs into five groups
- Collect previous science on each drug
- If science is insufficient then perform administrations on a minimum of 20 horses
- Plasma is preferred but some drugs will have both urine and plasma collected
- Ultimate goal...uniform withdrawal time with a corresponding concentration of drug which regulates the withdrawal time



Withdrawal Times Research

- | | |
|--------------------|--------------------|
| ■ Priority Group 1 | ■ Priority Group 2 |
| - Acepromazine | - Boldenone |
| - Butorphanol | - Dantrolene |
| - Detomidine | - Dexamethasone |
| - Glycopyrrolate | - Firocoxib |
| - Lidocaine | - Fluphenazine |
| - Mepivacaine | - Hydroxyzine |
| - Methocarbamol | - Nandrolone |
| - Pyrilamine | - Stanozolol |
| | - Testosterone |



Withdrawal Times Research

- | | |
|----------------------|--------------------|
| ■ Priority Group 3 | ■ Priority Group 4 |
| - Albuterol | - Atropine |
| - Betamethasone | - Beclomethasone |
| - Diclofenac | - Buscopan |
| - Methylprednisolone | - Cromolyn |
| - Reserpine | - Isoxsuprine |
| - Triamcinolone | - Pentoxifylline |
| - Trichlormethiazide | - Phenytoin |
| - Xylazine | - Prednisolone |



Model Rules-Present & Future Withdrawal Time Database

- Withdrawal times posted for 18 jurisdictions and over 70 drugs
- Database is searchable by drug and by jurisdiction
- Over 2,000 unique users in first month of availability
- www.rmtcnet.com, click on "Withdrawal Times" tab at top of page



RMTC-Present & Future Drug Testing Initiatives

- Develop strategies to improve drug testing in US:
 - Integrate WADA laboratory standards
 - Overhaul of Laboratory Quality Assurance Programs
 - Development of a post-doctoral recruitment program
 - Development of a business plan for U.S. drug testing infrastructure which includes research and development laboratory



Big picture...the future

- Beginning to see organizations with "sticks" use them to drive change:
 - Thoroughbred Owners and Breeders Association Graded Stakes Committee
 - Breeder's Cup
 - NTRA Safety Alliance
 - The Jockey Club Safety Committee
 - Hambletonian Society



	RMTC Pharmacokinetics and Drug Withdrawal Studies
	Scot Waterman, DVM Executive Director

	Terminology

	Definitions
	<ul style="list-style-type: none"> ■ Screening test- rapid and sensitive, specificity not crucial. A sample with a positive screening test would be said to be "suspicious" ■ Confirmation test- sensitive and specific, if the result is positive the presence of a forbidden substance is said to be confirmed.

	Definitions
	<ul style="list-style-type: none"> ■ Limit of Detection—the lowest concentration of a drug that can be detected by a particular laboratory method. Applies to screening and confirmation methodologies ■ Limit of Quantitation—the lowest concentration of a drug that can be reliably quantified by a particular laboratory method. Generally higher than the LOD and only relevant for confirmation methods

	Definitions
	<ul style="list-style-type: none"> ■ Withdrawal Time – the time period that must elapse from the time of administration of the last dose of a drug formulation until the drug concentration decreases below some concentration in urine and/or blood. <ul style="list-style-type: none"> - That concentration may be the LOD, the LOQ, or some predetermined concentration. - Factors affecting withdrawal time include dose, route of administration, formulation, number of doses administered, accuracy and precision of the assay, and within-horse and between-horse variability among other factors

	Definitions
	<ul style="list-style-type: none"> ■ Regulatory Limit <ul style="list-style-type: none"> - When a drug and/or its metabolites are found in the urine or blood at a concentration that is at or below the regulatory limit, no administrative action is taken. - Also called a "threshold" or "decision level" - European terminology = "screening limit" - Can be a LOD, LOQ, arbitrary, based on data from administration studies

Definitions

- **Zero Tolerance**—somewhat of a myth since no testing methodology can detect down to a concentration of zero.
 - Usually suggests that the laboratory notifies commission if any concentration of drug is detected
 - Entirely dependent on the method being used though

Zero tolerance?

Screening Method	Limit of Detection	Detection Time
Thin Layer Chromatography	~100 ng/ml	~24 hours
ELISA	~1 ng/ml	~72-96 hours
Mass Spectral Methods	~25 pg/ml	~7-30 days

Zero tolerance?

	Screening Method	Sample contains 150 ng/ml	Sample contains 50 ng/ml	Sample contains 50 pg/ml
State A	Thin Layer Chromatography	Positive	Negative	Negative
State B	ELISA	Positive	Positive	Negative
State C	Mass Spectral Methods	Positive	Positive	Positive

RMTC Studies

Objective

- Determine concentrations of various drugs and drug metabolites in test samples collected from exercise-conditioned horses to provide information for determining drug withdrawal times and performing pharmacokinetic studies.

Study Design

- Test substance
 - Blood - plasma
 - Urine
- Laboratory methods
 - LC-MS at lowest attainable limit of detection (LOD) using latest instrumentation and methodology
- Drug Administrations
 - Drugs were administered IV as single dose except for select drugs
 - Blood and urine samples were collected at specific times after drug administration - samples were sent to testing laboratories for analysis

Equine Performance Laboratory

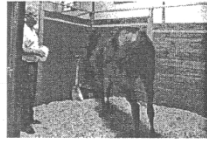
- Director
 - Pat Colahan, DVM
- Horses
 - Breeds
 - Thoroughbred
 - Standardbred (specific studies)
 - Gender
 - Geldings and mares
 - Age
 - 3-10 years
 - Fitness
 - Trained and assessed periodically

Equine Performance Laboratory

- Training and Fitness Assessment
 - Exercised 3 times per week on high-speed treadmill
 - Trot for 0.6 km at 4.0 m/s, gallop for 2 km at 8 m/s, and trot for 0.6 km at 4 m/s
 - Treadmill horizontal on Monday and inclined 6 degrees on Wednesday and Friday
 - Incremental Exercise Test to Exhaustion
 - Warm up on the treadmill at 4 m/s for 5 min before the start of the test; exercise for 1 min each at 9, 10, 11, 12, 13 and 14 m/s until they are unable to maintain the speed.
 - Blood samples collected for lactate and pH
 - Require lactate > 20 mM and pH < 7.35

Equine Performance Laboratory

- Sample collections
 - Blood - individual venipuncture into lithium heparin tubes - plasma harvested and stored at -70 °C
 - Urine - collected by free catch method - urine aliquotted and stored at -70 °C
- Sample distribution
 - Samples are sent by overnight courier to participating laboratories



RMTC Drug Projects in 2007-2008

Drug	PK Analyte	Withdrawal Analytes	Urine Analytes
Acepromazine	Acepromazine	Acepromazine + HEPS	HEPS
Butorphanol	Butorphanol	Butorphanol + conjugates	Not applicable
Detomidine	Detomidine	Detomidine + metabolites	Metabolites
Firocoxib	Firocoxib	Firocoxib + metabolites	Firocoxib + metabolites
Glycopyrrolate	Glycopyrrolate	Glycopyrrolate	Glycopyrrolate

RMTC Drug Projects in 2007-2008

Drug	PK Analyte	Withdrawal Analytes	Urine Analytes
Lidocaine	Lidocaine	Lidocaine + metabolites	Metabolites
Mepivacaine	Mepivacaine	Mepivacaine + metabolites	Metabolites
Methocarbamol	Methocarbamol	Methocarbamol	Not applicable
Pyrilamine	Pyrilamine	Pyrilamine + metabolites	Not applicable

RMTC Drug Projects in 2008

Drug	PK Analyte	Withdrawal Analytes	Urine Analytes
Boldenone	Boldenone	Boldenone	Total Boldenone
Nandrolone	Nandrolone	Nandrolone	Total Nandrolone
Stanozolol	Stanozolol	Stanozolol	Total 16-hydroxystanozolol
Testosterone	Testosterone	Testosterone	Total Testosterone

RMTC Drug Projects in 2009

Drug	PK Analyte	Withdrawal Analytes	Urine Analytes
Albuterol	Albuterol	Albuterol + total albuterol	Total albuterol
Dantrolene	Dantrolene	Dantrolene + 5-hydroxydantrolene	Total 5-hydroxydantrolene
Fluphenazine	Fluphenazine	Fluphenazine	Not applicable
Hydroxyzine	Hydroxyzine + cetirizine	Hydroxyzine + cetirizine	Hydroxyzine + cetirizine
Xylazine	Xylazine	Xylazine + 4'-hydroxyxylazine + 3'-hydroxyxylazine	Xylazine + metabolites

Process for sample analysis

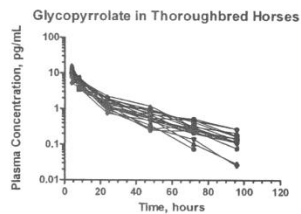
- Use accredited laboratories (ISO 17025).
- Use LC-MS or LC-MS-MS.
- Develop and validate methods for each analyte before testing experimental samples.
- Schedule sample collection for a drug study after the proposed method has been suitably validated and approved for use in the study.

Glycopyrrolate Example

- Quaternary amine
- Anti-cholinergic
- Popular pre-anesthetic but used in racing as a respiratory "aid" due to bronchodilatory effect and reduction in airway secretions
- Rapidly eliminated making detection more difficult and closer to race time administration possible

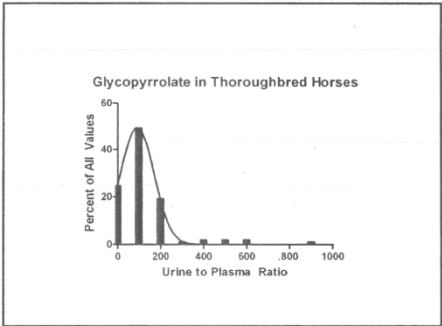
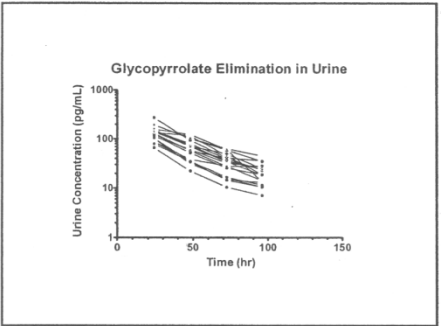
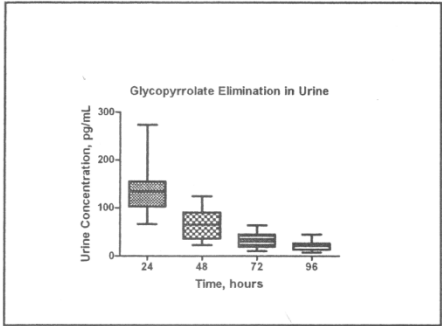
Glycopyrrolate Example

- One horse pilot study performed
- LC/MS/MS optimized to LOD in sub-picogram concentrations
- 20-horse administration using 1 mg/horse IV and 10 mg/horse orally (n=6)
- Sample analysis on LC/MS/MS TSQ Quantum Ultra at University of Florida
- Results calculated using Prism



Plasma Threshold

Time, hours	Threshold concentration based on 99/95 Tolerance Interval
24	12.5 pg/mL
48	1.3 pg/mL
72	0.63 pg/mL
96	0.33 pg/mL





NEWS RELEASE

October 1, 2009
Contact: Hallie Lewis (859) 224-2848

RMTC BOARD FORMS COMMITTEE TO OVERSEE IMPLEMENTATION OF DTI TASK FORCE RECOMMENDATIONS

Moving forward with its initiative to significantly change the structure of drug testing in U.S. horse racing, the Racing Medication and Testing Consortium (RMTC) board of directors has approved the formation of a committee to oversee the implementation of the Drug Testing Initiative (DTI) Task Force recommendations on quality assurance and laboratory accreditation programs for U.S. horse racing drug testing laboratories.

The board took this action at its meeting in Louisville, Ky., on September 29, 2009.

Named to this committee, chaired by Thoroughbred Horsemen's Association CEO Alan Foreman, were RMTC board members:

- Gary Carpenter, American Quarter Horse Association executive director of racing
- Dr. Robert Lewis, past president of the American Association of Equine Practitioners
- Ed Martin, president of the Association of Racing Commissioners International
- Dan Metzger, president of the Thoroughbred Owners and Breeders Association
- Terry Meyocks, national manager of the Jockeys' Guild
- Dr. Gregg Scoggins, Magna Entertainment Corp.'s national director of regulatory affairs
- Dr. Scott Stanley, UC Davis associate professor
- Kent Stirling, Florida HBPA executive director
- Mike Tanner, executive vice president of the United States Trotting Association
- Mike Ziegler, executive director of the NTRA Safety and Integrity Alliance

At the board meeting, other integrity issues were also discussed in depth, including the use of non-steroidal anti-inflammatory drugs (NSAIDs) and how they may affect pre-race examinations by regulatory veterinarians. Dr. Tom David of the Louisiana State Racing Commission and chairman of the ARCI Racing Regulatory Veterinarian Committee explained the examining veterinarian's concern over current NSAID and corticosteroid policies. The RMTC Scientific Advisory Committee was previously charged with the task of reviewing all existing research on NSAIDs, particularly phenylbutazone.

Dr. Larry Soma of the University of Pennsylvania recently prepared an extensive report and has concluded that "based on scientific reports and the impression of clinical veterinarians, residual effects of phenylbutazone remain at 24 hours."

not rise to the level of an attempt to illegally affect the outcome of a race, and there needs to be a better system to communicate and differentiate racing stable staff management errors from attempts to drug a horse in order to win a race,” said RCI President Ed Martin.

- RMTC’s DTI Task Force, which is funded in large part by The Jockey Club, announced that applications for the graduate/post-doctoral research fellowship program are being mailed to university-affiliated laboratories by October 1. Funding from RMTC is available for one person, with the respective university matching costs. The RMTC board previously committed \$75,000 per year for three years for this program. Applications are due January 1, 2010, and an offer will be made April 1, following the review process. This program is intended to develop the next generation of horse racing laboratory directors and researchers.
- RMTC is also actively seeking applications for the new position of Director of Research and Accreditation. The job will involve coordinating administration studies designed for the development of uniform threshold levels and withdrawal times for the regulation of therapeutic medication, analyzing data produced from those studies and producing a final written report for distribution to the Scientific Advisory Committee, assisting principal investigators with publication of administration studies in peer review journals, and coordinating the development of an RMTC/RCI laboratory accreditation and quality assurance program, among other duties. A complete job description can be found at rmtcnet.com. Applications are being sought immediately with the intention to fill the position before the end of the year.

Mike Tanner of the United States Trotting Association appreciated the progress that RMTC board members made at this crucial meeting.

“We covered a good amount of ground Tuesday and have targeted some very important issues, especially uniform medication and testing protocols,” he said. “Now it’s just a matter of getting to that point. There’s still a lot of work to be done, but I think we’re all on the same page and I’m encouraged by the steps that are being taken.”

The RMTC, which is based in The Jockey Club’s Kentucky office, consists of 25 racing industry stakeholders and organizations that represent Thoroughbred, Standardbred, American Quarter Horse and Arabian racing. The organization works to develop, promote and coordinate, at the national level, policies, research and educational programs that seek to ensure the fairness and integrity of racing, the health and welfare of racehorses and participants, and to protect the interests of the racing public.

For additional information, visit the RMTC website at rmtcnet.com or contact Hallie Lewis, RMTC director of communications, at (859) 224-2848.

The RMTC Scientific Advisory Committee has identified several options for consideration in future recommendations on the administration times and threshold concentrations for NSAIDs. California Horse Racing Board Equine Medical Director Dr. Rick Arthur explained that several states are participating in a study to evaluate NSAID concentrations in blood at the time of pre-race examinations. The Scientific Advisory Committee will assess this data and make a final recommendation at the next RMTC board meeting.

The RMTC previously announced that it was conducting research on corticosteroids commonly used in racing and the board was updated on the status of that project. A copy of Dr. Soma's report and Dr. David's comments can be obtained at the RMTC website (rmtcnet.com).

In other RMTC business:

- Dr. Rick Sams of the University of Florida Racing Laboratory presented to the board a report on administration studies for a number of commonly used therapeutic medications that often result in medication infractions. Dr. Sams also announced that the administration studies on anabolic steroids are complete and a recommendation on a model rule for testing in plasma for these medications has been submitted to RCI.
- * Administration studies that are nearing completion of sample analysis are acepromazine, butorphanol, detomidine, glycopyrrolate, lidocaine, mepivacaine, methocarbamol and pyrilamine.

"RMTC anticipates that by early next year, it will be in a position to make recommendations for threshold levels and/or withdrawal times on these medications," said RMTC Chairman Dr. Robert Lewis. "Most therapeutic medication infractions are simple management mistakes. Our hope is this information will help veterinarians and trainers avoid these common rule violations."

This announcement was met with appreciation by Florida HBPA Executive Director Kent Stirling. "Horsemen will be glad to see that we are finally going to have thresholds and withdrawal times for these medications," said Stirling. "The withdrawal times database on the RMTC website is extremely helpful but shows the inconsistencies in withdrawal times recommended by state racing commissions and veterinarians. The recommendations out of these research studies will certainly help to bring about uniformity, which is a primary mission of RMTC and something that horsemen desperately want to see realized."

- The RMTC board also approved the formation of a Communications Subcommittee to make recommendations on a media campaign to better explain the significant efforts of the drug testing laboratories in detecting prohibited drugs, and differentiate those findings from the more prevalent positive laboratory reports for overages of approved therapeutic medication that are primarily mistakes in administration times and dosages.

"Judging from the media reports and comments on many blogs, it is clear that many reporters and particularly racing fans do not understand that an overage of flunixin does

From: Rick Sams [RSams@hflss.com]
Sent: Monday, February 06, 2012 2:13 PM
To: Lynn Hovda
Subject: RE: Acepromazine or Promazine Sulfate

Hi Lynn –

I have seen Scot's stuff with the 25 ng/mL threshold but I do not know its origins. We used 10 ng/mL in Florida after we had demonstrated that this concentration afforded adequate control of acepromazine for a period of about 24 hours. Higher concentrations permitted closer use. Scott Stanley's work at UC Davis for the RMTC is going to show that the 10 ng/mL threshold is appropriate. The threshold applies to the total concentration of 2-(1-hydroxyethyl)promazine sulfoxide (HEPS). The metabolite can be purchased commercially and appropriate internal standards are available.

Sincerely,
Rick

Rick Sams
Lab Director

HFL Sport Science

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From: Lynn Hovda [mailto:LHovda@mnrace.com]
Sent: Saturday, February 04, 2012 1:22 PM
To: Rick Sams
Subject: Acepromazine or Promazine Sulfate

Rick,

I am wondering if you can clarify where the RMTC is going...or has already gone.. in regards to testing for acepromazine. When Scot Waterman was here in November 2010 he provided the MRC with a level of 25 ng/ml (urine) for acepromazine yet I see that the Jockey Club has proposed a level of 10 ng/ml (urine) for the major metabolite, promazine sulfate.

Can you shed some light? And as always, thank you ever so much.

Lynn

Lynn Hovda, RPH, DVM, MS, DACVIM
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Pharmacokinetics and disposition of clenbuterol in the horse

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Soma, L. R., Uboh, C. E., Guan, F., Moate, P., Luo, Y., Teleis, D., Li, R., Birks, E. K., Rudy, J. A., Tsang, D. S. Pharmacokinetics and disposition of clenbuterol in the horse. *J. vet. Pharmacol. Therap.* 27, 71–77.

The pharmacokinetics of clenbuterol (CLB) following a single intravenous (i.v.) and oral (p.o.) administration twice daily for 7 days were investigated in thoroughbred horses. The plasma concentrations of CLB following i.v. administration declined mono-exponentially with a median elimination half-life ($t_{1/2k}$) of 9.2 h, area under the time–concentration curve (AUC) of 12.4 ng·h/mL, and a zero-time concentration of 1.04 ng/mL. Volume of distribution (V_d) was 1616.0 mL/kg and plasma clearance (Cl) was 120.0 mL/h/kg. The terminal portion of the plasma curve following multiple p.o. administrations also declined mono-exponentially with a median elimination half-life ($t_{1/2k}$) of 12.9 h, a Cl of 94.0 mL/h/kg and V_d of 1574.7 mL/kg. Following the last p.o. administration the baseline plasma concentration was 537.5 ± 268.4 and increased to 1302.6 ± 925.0 pg/mL at 0.25 h, and declined to 18.9 ± 7.4 pg/mL at 96 h. CLB was still quantifiable in urine at 288 h following the last administration (210.0 ± 110 pg/mL). The difference between plasma and urinary concentrations of CLB was 100-fold irrespective of the route of administration. This 100-fold urine/plasma difference should be considered when the presence of CLB in urine is reported by equine forensic laboratories.

(Paper received 20 August 2003; accepted for publication 12 January 2004)

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INTRODUCTION

Clenbuterol (CLB) is used for the management of acute and chronic respiratory disorder in horses (Dixon, 1992; Erichsen *et al.*, 1994; Mair, 1996), but its disposition in horses has not been thoroughly investigated.

Using gas chromatography for analysis, a single i.v. dose of CLB (1.6 µg/kg), produced concentrations of CLB in urine that were below the level of quantification (LOQ) 12 h postadministration (Collett *et al.*, 1983). However, when high performance liquid chromatography (HPLC)/enzyme-linked immunosorbent assay (ELISA) was used CLB was detected in plasma up to 24 and in urine 96 h following i.v. administration of 0.8 µg/kg.

Following oral administration of the same dose to horses, confirmation of the presence of CLB by GC/MS was reported at 48 and 75 h in plasma and urine samples, respectively (Dumasia & Houghton, 1991; Hagedorn *et al.*, 1995). With pre-extraction of urine prior to screening by ELISA, CLB concentrations of between 100 and 1000 pg/mL were detected and confirmed (Harkins *et al.*, 2000). Metabolites of CLB have been described in a number of species (Kopitar & Zimmer, 1976b; Kopitar & Zimmer, 1976a; Zalko *et al.*, 1998a; Zalko *et al.*, 1998c). Two hours following oral administration of 0.8 µg/kg BID for

5.5 days, the plasma concentration of CLB was between 0.45 and 0.75 ng/mL and the mean beta half-life was 10.4 h and plasma concentrations were below the LOQ in 3 days post CLB administration (Kallings *et al.*, 1991; Kleemann *et al.*, 1999). Serum CLB declined to 30 and 10 pg/mL at 48 and 72 h, respectively following the administration of 0.8 µg/kg. Serum concentrations of CLB were below the level of detection at 96 h (Lehner *et al.*, 2001).

The specific aim of this study was to determine the pharmacokinetics and urinary excretion of an intravenous and multiple oral administrations of CLB in the horse.

MATERIALS AND METHODS

Experimental animals

Eight thoroughbred mares, 4–10 years old and weighing 566.8 ± 50.4 kg, were used to complete six i.v. and six multiple oral administrations. Sequence of drug administrations was on a random basis. Horses were brought into stalls 2 days before the experiment started and remained housed for the duration of the study. They were fed grass hay and water *ad libitum*. All horses

were no longer actively racing but were otherwise in good health. Four horses were used for both the oral and i.v. study, but due to injury and illness two new horses had to be used to complete the oral study. The University of Pennsylvania Institutional Animal Care and Use Committee approved the protocol.

Dosage

Horses were administered CLB 1.6 µg/kg intravenously (i.v.) (Ventipulmin™ Solution; Boehringer Ingelheim, Burlington, Ontario, Canada) or orally (p.o.) (Ventipulmin™ Syrup; Boehringer Ingelheim, St Joseph, MO, USA) with a minimum 30-day washout period between administrations.

Intravenous administration

Blood samples were collected using a 14-F catheter (Angiocath, Becton Dickinson, Sandy, UT, USA) placed in the jugular vein before drug administration (zero time) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 6, 8, 12, 16, 20, 24, 28, 32, 36, 48, 72, and 96 h after CLB administration. Heparin was used as the anticoagulant. A contra-lateral jugular vein was used for i.v. drug administration. The samples were centrifuged (2500 *g* for 15 min) to obtain plasma. Aliquots of plasma were immediately frozen at -20 °C and later stored at -70 °C until analyzed. Each aliquot of sample was used once to eliminate any effect of freeze-thaw cycles on the concentration of CLB in the samples. An indwelling 24-F self-retaining catheter (Foley Catheters; CR Bard Inc., Covington, GA, USA) placed in the bladder and attached to a drainage bag (Bard Center Entry Urinary Drainage Bag; CR Bard Inc., Covington, GA, USA) was used for continuous collection of urine. The total volume of urine collected at 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 28, 32, and 36 h after drug administration was measured. Urine samples were also collected at 48, 72, and 96 h. The samples were divided into aliquots, immediately frozen at -20 °C and then stored at -70 °C. The total i.v. dose was 892.3 ± 44.9 µg.

Oral administration

CLB was administered orally (BID, at 7 AM and 7 PM) for 7 days. Control plasma and urine were collected before drug administrations (day 0). Plasma samples were collected 6 h after the first oral dose and at each morning immediately prior to the 7 AM administration and at 6 h following the administration. Following the last AM dose of CLB on day 7, blood samples were collected at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 6, 8, 12, 16, 20, 24, 28, 32, 36, and thereafter, daily for up to 288 h (12 days). Urine samples were collected at 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 28, 32, 36, 48 h and thereafter every 48 h for up to 288 h post CLB administration. The total p.o. dose was 886.3 ± 85.6 µg.

Quantification of CLB in equine plasma and urine

Clenbuterol was recovered from plasma and urine samples by liquid-liquid extraction, separation of CLB was by liquid

chromatography on a short cyano column (Zorbax SB-CN, 2.1 × 50 mm, 5 µm with a SB-C3 guard column, 2.1 × 12.5 mm, MAC-MOD Analytical, Chadds Ford, PA, USA) and pseudo multiple reaction monitoring (pseudo-MRM) by electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-QTOF-MS/MS; Micromass, Beverly, MA, USA). Mabuterol hydrochloride (Boehringer Ingelheim, Ingelheim am Rhein, Germany) was used as the internal standard. The LOQ for CLB in plasma was 13 pg/mL and 50 pg/0.1 mL of urine. Extraction ratio using petroleum ether was 71.7% (Guan *et al.*, 2002).

Pharmacokinetic analysis

I.v. administration

Plasma concentration vs. time data following i.v. administration of a dose ($D_{i.v.}$) of CLB was analyzed by nonlinear least-squares regression analysis (WinNonlin Version 4.0; Pharsight Corp., Mountain View, CA, USA). Mono-exponential and bi-exponential equations were fitted to the data from each horse. The number of exponential terms and the weighting function required to describe the data were based on the appearance of the curve, the Akaike information criterion, and the *F*-test (Akaike, 1973, 1976; Gabrielsson & Weiner, 2000). The weighting factor was $1/(Y_{hat} \times Y_{hat})$. A mono-exponential equation best described the data from each horse.

$$C_p^t = Ae^{-kt}$$

where A was the coefficient, k the elimination rate constant, and C_p^t was the plasma concentration of CLB at specific time points (t). Half-lives were calculated as the natural log (base₂) divided by the rate constant.

Volume of the central compartment (V_c) was calculated from

$$V_c = \frac{D_{i.v.}}{C_p^0}$$

Clearance (Cl) was calculated as

$$Cl = V_d \times k$$

The total amount of the i.v. dose eliminated unchanged in urine (X_u) from 0 to 36 h was calculated from

$$X_u = \sum (V_u \times C_u)$$

where V_u was the volume of urine and C_u the concentration of CLB in urine.

Multiple oral administrations

Plasma concentration vs. time data following p.o. administration of CLB was analyzed by a nonlinear least-square regression (Boston *et al.*, 1981) (WinSaam, National Institutes of Health, Bethesda, MD, USA). Mono-exponential and bi-exponential equations were fitted to the terminal plasma concentrations from each horse. The number of exponential terms and the weighting function required to describe the data were based on the appearance of the curve, the percent reduction in the sums of squares and the minimization of fractional standard deviation of

the parameters (Stefanovski *et al.*, 2003). The weight applied was in the form of $W = 1/SD^2$ of the observed data (Wastney *et al.*, 1999).

The compartmental model developed from the i.v. administration studies was modified to describe the absorption and elimination phase of orally administered CLB. For the analysis of the oral absorption kinetics, the V_d was assumed to be the similar as that determined in the i.v. investigation for the corresponding horse. Since two of the oral investigations were carried out on horses that did not undergo i.v. investigations, the V_d for these two horses were assumed to be the mean V_d of the four horses that had undergone i.v. investigations. This approach assumes that the V_d for a particular horse is relatively constant from day to day and not influenced by the route of administration of the drug. On the other hand in the model to describe the oral absorption kinetics of the last dose CLB, we allowed the clearance parameter to vary (i.e. determined the 'best fit estimate' of this parameter) on the assumption that clearance rate of drug during the oral investigation could well differ from that determined during the i.v. investigation. A 15 min delay period was incorporated into the model to allow for the transit of CLB from the stomach to its appearance in plasma. This delay was based on the first sampling period following the administration (Fig. 1b). Absorption of CLB was so rapid following multiple administrations and the concentration peaked quickly, that there were insufficient data points to allow the complete description of the absorption phase.

Normally, in order to estimate the proportion of the oral dose absorbed, the $AUC_{p.o.}/AUC_{i.v.}$ is calculated following the normalization of the oral dose with respect to the i.v. dose. In the multiple oral dose investigations precluded the use of this method. Instead, using the absorption model shown in Fig. 1, the absorption percentage (F) for the oral dose is estimated directly by the least squares 'best fit' of the model to the data as

$$P = 100 \times k_{2,1}/(k_{2,1} + k_{2,0})$$

The terminal urine excretion following the last p.o. dose was best described by appearance and bi-exponential elimination equation, where A, B, and C were the appearance, and terminal coefficients λ , α , and β were the appearance and terminal exponents.

The area under the plasma concentration vs. time curve (AUC) for the i.v. administration and the terminal portion of the

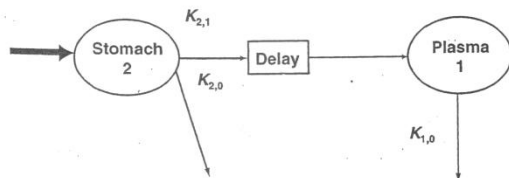


Fig. 1. Illustration of the p.o. absorption and elimination model. The fraction of CLB absorbed was calculated as $k_{2,1}/k_{2,1} + k_{2,0}$. Delays were added based on appearance of CLB in plasma from the stomach.

multiple p.o. administrations was calculated by the trapezoidal rule. The AUC for the iv administration was calculated from 0 to 48 h and for the multiple p.o. administration from 168 to 240 h (96 h) which represented the terminal portions of the plasma concentration vs. time curve following the last p.o. administration.

Statistical analysis

Pharmacokinetic parameter estimates of CLB were expressed as median and range and the nonparametric Wilcoxon and Kruskal-Wallis rank-sum tests were used for statistical comparisons of parameters (JMP Version 4.0; SAS Institute Inc., Cary, NC, USA) (Powers, 1990). ANOVA was used for parametric analyses. The plasma and urine concentrations of CLB were expressed as mean (\bar{X}) and standard deviation (SD). Significance was designated at $P < 0.05$.

RESULTS

Plasma concentrations

Following bolus i.v. administration of CLB, a mono-exponential equation best described the data for each horse (Fig. 2). The median and range of the pharmacokinetic parameter estimates for the i.v. administration are shown in Table 1. Plasma concentrations of CLB were quantifiable in all six horses at 48 h. At 72, five of six horses and at 96 h two of six horses, CLB

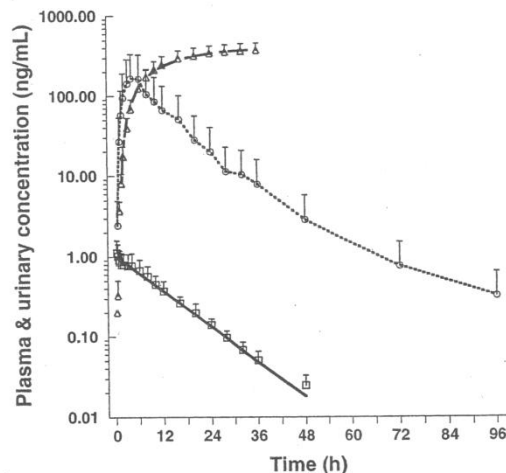


Fig. 2. Plasma (\square) and urine (\circ) concentrations of CLB ($\bar{X} \pm SD$) following the i.v. administration of 1.6 $\mu\text{g}/\text{kg}$. Lines of best fit for plasma (solid) and the mean urine concentration (dashed). The accumulative urinary excretion of CLB (Δ). The urinary concentration was ~ 100 -fold greater than that of plasma at and after the peak period of urinary excretion ($n < 6$ horses).

Table 1. Pharmacokinetic parameter estimates (median and range) following a single i.v. administration (1.6 µg/kg) of CLB (n = six horses)

Parameters	Median	Range
A (ng/mL)	1.04	0.50–1.43
k (/h)	0.072	0.062–0.082
t _{1/2k} (h)	9.2	8.4–11.1
Cl (mL/h/kg)	120.0	81.4–199.1
AUC _(0,48) (ng·h/mL)	12.4	8.2–15.9
V _d (mL/kg)	1616.0	1118.8–3184.4

A, coefficient; k, exponent; t_{1/2k}, elimination half-life; Cl, total clearance; AUC, area under the curve (0–48 h); V_d, volume of distribution.

was detected but not quantified because the concentrations were below the LOQ. The plasma concentrations were 1126.6 ± 483.0, 140.0 ± 26.8, 50.6 ± 8.4, and 24.6 ± 8.4 pg/mL, at 0.25, 24, 36, and 48 h, respectively.

The terminal plasma concentration time curve of CLB following multiple p.o. administrations (1.6 µg/kg, BID for 7 days) was also best described by mono-exponential equation (Fig. 3). The median and range of the pharmacokinetic parameter estimates of the multiple p.o. administration are shown in Table 2. A significant difference was noted (P < 0.01) only in the k parameter estimate when comparing the i.v. and p.o. administration.

Following the multiple p.o. administrations the plasma concentrations of CLB were quantifiable in all six horses at

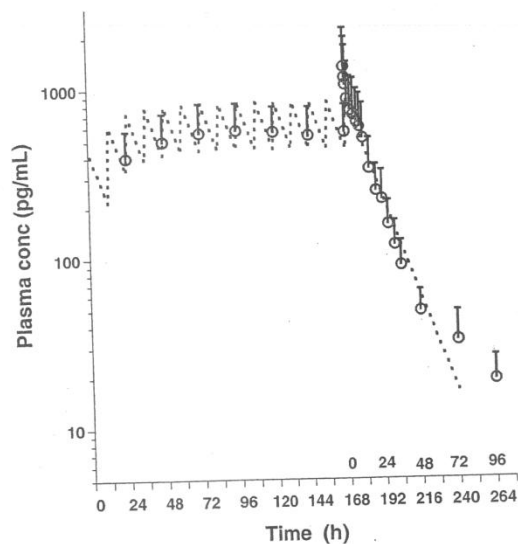


Fig. 3. The daily AM plasma concentrations (○) ($\bar{X} \pm SD$) following the p.o. administration of clenbuterol (1.6 µg/kg) twice a day for 7 days and the 96 h terminal elimination concentrations. The dotted line is modeled peak and trough concentrations and line of best fit for the terminal elimination phase.

Table 2. Pharmacokinetic parameter estimates (median and range) following p.o. administrations of CLB 1.6 µg/kg BID for 7 days (n = six horses)

Parameters	Median	Range
k (/h)	0.054	0.046–0.063
t _{1/2k} (h)	12.9	11.0–15.0
Cl (mL/h/kg)	94.0	52.7–190.4
AUC _(168,264) (ng·h/mL)	18.6	7.4–26.3
V _d (mL/kg)	1574.7	1142.4–3321.9
F (%)	83.3	68.3–87.7*

k, exponent; t_{1/2k}, elimination half-life; Cl, clearance; AUC, area under the curve (168–264 h); V_d, volume of distribution; F, fraction absorbed. *n = five horses only.

72 h. At 96 h in three of six horses CLB was detected but concentrations were below the LOQ. The 72 and 96 h plasma concentrations suggest a second compartment, which could not be adequately described.

The plasma concentration of CLB at 24 h following the initial 7 AM and 7 PM administrations was 340.7 ± 131.1 pg/mL. This 24 h plasma concentration was significantly different (P < 0.003) from the subsequent 7 AM samples collected 48–168 h (508.1 ± 249.4 pg/mL). Significant differences did not exist between plasma concentrations measured at 7 AM from 48–168 h. Based on this observation an apparent steady-state of CLB in plasma was reached within 48 h of the start of the p.o. administrations.

Samples were also collected daily 6 h following the 7 AM p.o. administration (24–168 h). These midday plasma concentrations were 679.3 ± 243.9 ng/mL and were all significantly higher (P < 0.0003) than the corresponding 7 AM plasma concentrations. The last oral administration rapidly increased from the 7 AM baseline CLB plasma concentration of 537.5 ± 268.4 to a peak concentration of 1302.6 ± 925.0 pg/mL at 0.25 h (Fig. 4). The concentration of CLB subsequently declined to 220.6 ± 125.3, 48.1 ± 15.8, 33.4 ± 14.5, and 18.9 ± 7.4 pg/mL at 24, 36, 48, 72, and 96 h, respectively.

Urine concentrations

The peak concentration of CLB in urine following the i.v. administration was 176.3 ± 54.9 ng/mL at 4.9 ± 1.3 h (Fig. 2). The total CLB accumulated urinary excretion at 36 h was 372.8 ± 83.4 µg, or 42.1 ± 10.6% of the total i.v. dose of 892.3 ± 49.9 µg.

Clenbuterol concentration in urine following the last p.o. administration on day 7 increased from 52.1 ± 12.4 ng/mL to a peak of 113.4 ± 47.6 ng/mL at 6.3 ± 3.4 h. Urinary CLB was still quantifiable in three of six horses at day 12 (0.21 ± 0.11 ng/mL) following the last administration of CLB to horses and was still present in the urine samples 8 days beyond the last quantifiable CLB in plasma.

The urine appearance t_{1/2λ} following the last p.o. administration was 1.4 h (0.7–2.2) reaching a maximum concentration

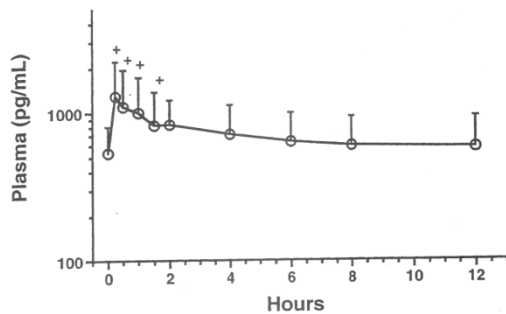


Fig. 4. Changes in plasma concentrations of CLB ($\bar{X} \pm \text{SD}$) during the first 12 h following the last p.o. administration of 1.6 $\mu\text{g}/\text{kg}$ on day 7. There were rapid increases in plasma concentrations from AM baseline plasma concentrations. These increases were significant from the baseline ($P < 0.004$) between 0.25 and 1 h (+) following the last p.o. administration ($n = \text{six horses}$).

of $102.2 \pm 33.6 \text{ ng/mL}$ at $4.3 \pm 1.2 \text{ h}$. The $t_{1/2\alpha}$ and $t_{1/2\beta}$ of the elimination phase were 11.8 h (7.9–13.7), and 64.7 h (52.6–90.5), respectively.

In comparing CLB concentrations, there was a 97.3 ± 9.9 and 99.0 ± 1.0 -fold difference between the urinary and plasma concentrations for the i.v. and p.o. administrations, respectively. Thus, following the peak appearance of urine, the concentration of CLB in urine was approximately 100-fold greater than that in plasma, regardless of the route of administration.

DISCUSSION

The chemical structure of the β_2 -adrenoceptor agonist CLB consists of a catechol ring or related group, which is important primarily for its potency and an ethanolamine side chain that is responsible for selectivity of action (Popa, 1986). The selectivity refers to the affinity of the drug molecule for the β_1 or β_2 receptors. Most of the catechols are metabolized by *O*-methylation, and those with a phenolic hydroxyl group are readily metabolized to the *O*-sulfate ester conjugate. The exception to this rule is CLB, where the phenolic hydroxyl groups have been replaced with 3, 5-dichloro-4-amino substitution. This substitution promotes greater resistance to sulphation and accounts for the lower metabolic clearance, high systemic availability, and a decreased first-pass metabolism. This renders CLB more suitable for oral use than other β_2 -adrenoceptor agonists (Popa, 1986; Morgan, 1990).

The pharmacokinetics and metabolism of CLB have been investigated in a number of species including man, rat, dog, and rabbit and a biphasic elimination pattern was described following oral administration with a beta half-life of 9–35 h (Kopitar & Zimmer, 1976a; Zimmer, 1976a, b; Yamamoto *et al.*, 1985). Metabolites of CLB were detected in minor concentrations in urine extracts (Zimmer, 1976a; Zalko *et al.*, 1998a, c). More than 70–80% of the radioactivity was eliminated from plasma of

the rat and the dog within the first 24 h and less than 5% was by fecal excretion (Kopitar & Zimmer, 1976a).

The pharmacokinetics of many of the β_2 -agonists have not been well described because of limitations in the quantification of low concentrations of these agents in plasma. Radio-labeled CLB, enzyme immunoassays, and the lesser sensitive GC/MS methods have been used in pharmacokinetic and metabolism studies in laboratory animals, human and horses (Kopitar & Zimmer, 1976a; Zimmer, 1976a, b; Collett *et al.*, 1983; Dumasia & Houghton, 1991; Kallings *et al.*, 1991; Hagedorn *et al.*, 1995; Kleemann *et al.*, 1999; Harkins *et al.*, 2000).

Clenbuterol is a basic compound with its basicity resulting from a tert-butylamino group in the side chain. The pK_a of CBL is estimated to be greater than 10, based on the fact that the pK_a of tert-butylamine is 10.83 and that of dimethylamine is 10.73 (Weast, 1973). At the physiological pH (~ 7.4), CBL exists in its unionized form and is water soluble.

The absorption phase of the last p.o. administration of CLB following the multiple oral administrations was rapid, therefore the absorption phase could not be defined (Fig. 4). To allow for the absorption of CLB from the stomach to plasma a delay was incorporated into the model (Fig. 1). The rapid increase in baseline from 537 to 1302 ng/mL suggests an equilibration of tissues had occurred at the administered concentration with a reduced uptake by body tissues. The oral mono-exponential $t_{1/2}$ of 12.9 h and a dosing interval of 12 h indicate an accumulation ratio of ~ 2 (Baggot, 1977). The time to reach a steady-state is roughly three to four times the terminal half-life, data present indicated that a steady-state occurred in 48 h.

The terminal portions of plasma concentration of CLB following oral administration were also described by a first-order exponential equation. The median $t_{1/2k}$ for the 7-day multiple administrations of CLB was 12.9 h, which was longer than 9.2 h for the single i.v. administration group. There were no significant differences between the i.v. and p.o. administration pharmacokinetic parameter estimates, with the exception of the $t_{1/2k}$ estimation. The 72 and 96 h concentrations of CLB in plasma suggested the start of a much slower phase of elimination, but the LOQ disallowed any use of concentrations of CLB measured beyond 96 and concentrations were not measured in all horses at 96 h. The quantification of CLB in urine for 8 days beyond the last measured plasma concentration indicates a slower elimination, which could not be well defined in plasma. The modeling of the urine following the last dose showed a terminal β phase of $\sim 65 \text{ h}$ and α phase of $\sim 12 \text{ h}$, indicating a slower elimination which could not be defined because of the LOQ of CLB in plasma. There was no significant difference between the plasma $t_{1/2k}$ and $t_{1/2\alpha}$ of urine. Studies in agriculture animals, tissue concentrations were highest in the eye, liver, kidney, (Sauer *et al.*, 1995; Petkova & Stanchev, 1996; Smith & Paulson, 1997; Zalko *et al.*, 1998a). Horses administered ^{14}C -CLB BID daily for 21 days (0.8 $\mu\text{g}/\text{kg}$), the highest concentrations of radioactivity were in the liver and kidney with the lowest in fat tissue (Johnston & Dunsire, 1996). These high residual concentrations in liver may contribute to the persistent concentrations found in urine as the liver is cleared of CLB.

Estimate for F in five horses was 83.3%. In one horse the F was of 26.4%. This low value was unexplained and considered an outlier, as all horses were managed in a similar manner. Plasma concentrations of this horse were low throughout the study indicating low absorption through the study.

The 100-fold difference between plasma and urinary concentrations of CLB, the lack of metabolism and the sustained presence of CLB in urine 8 days beyond the last quantifiable concentration of CLB in plasma account for the ability of equine forensic laboratories to detect and confirm the presence of CLB in urine for many days after CLB had been withdrawn. Thus, the relationship between the plasma and urinary concentrations of CLB is an important observation and should be considered when determining the pharmacologic significance of detecting CLB in post race urine samples. This difference also suggests that plasma is more suitable to determine a reasonable withdrawal time following the last dose.

None of the studies available to date have determined the specific duration of pharmacological actions relative to plasma concentration of CLB (Shapland *et al.*, 1981; Boner *et al.*, 1987a, b). A BID administration of CLB is recommended for proper maintenance of horses with chronic obstructive pulmonary disease and the dose is dependent upon the severity of the disease.

The dose commonly used for horses under racing conditions is 0.8–1.6 µg/kg. Following a dose of 1.6 µg/kg, the average 24 h plasma concentration of CLB was ~500 pg/mL. The use of plasma clearance and the dose to calculate an effective plasma concentration (EPC), a method based on several assumptions, suggests that ~500 pg/mL as a therapeutically effective dose (Toutain & Lassourd, 2002). These authors convert this to an irrelevant plasma concentration by using a safety factor of 500, which becomes 1 pg/mL and a ~10 day withdrawal time.

At 72–96 h the concentration of CLB in plasma had declined to ~35 and ~20 pg/mL, respectively which is a reduction of approximately 96–97%. Thus, these low concentrations of CLB at 72–96 h postadministration should be considered a reasonable withdrawal time. The result of this study also suggested that the plasma rather than urine CLB concentrations should be used for forensic purposes.

ACKNOWLEDGMENTS

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REFERENCES

- Akaike, H. (1973) A new look at statistical model identification. *Transactions Automatic Control*, **19**, 716–723.
- Akaike, H. (1976) An information criterion (AIC). *Mathematical Science*, **14**, 5–9.
- Baggot, J.D. (1977) *Principles of Drug Disposition in Domestic Animals: Basis of Veterinary Clinical Pharmacology*. pp. 204. W.B. Saunders, Philadelphia, PA.
- Boner, A.L., Bennati, D., Castellani, C., Sette, L. & Schiassi, M. (1987a) Bronchodilating activity of oral clenbuterol in asthmatic children after single administration of different dosages. *Pediatric Pulmonology*, **3**, 34–37.
- Boner, A.L., Sette, L., Castellani, C. & Schiassi, M. (1987b) Oral clenbuterol and procaterol. A double-blind comparison of bronchodilator effects in children with chronic asthma. *Journal of Asthma*, **24**, 347–353.
- Boston, R.C., Greif, P.C. & Bertram, M. (1981) Conversational SAAM: an interactive program for kinetic analysis of biological systems. *Computer Programming Biomedical*, **13**, 111–119.
- Collett, L., Kamerling, S., Woods, W.E., Weckman, T., Nugent, T., DeQuick, D. & Tobin, T. (1983) Clearance times and behavioral effects of clenbuterol in the horse. *Proceedings of the 5th International Conference of Cont. Use Drugs Racehorses*, Toronto, Canada.
- Dixon, P.M. (1992) Respiratory mucociliary clearance in the horse in health and disease, and its pharmaceutical modification. *Veterinary Record*, **131**, 229–235.
- Dumasia, M.C. & Houghton, E. (1991) Screening and confirmatory analysis of beta-agonists, beta-antagonists and their metabolites in horse urine by capillary gas chromatography-mass spectrometry. *Journal of Chromatography A*, **564**, 503–513.
- Erichsen, D.F., Aviad, A.D., Schultz, R.H. & Kennedy, T.J. (1994) Clinical efficacy and safety of clenbuterol HCl when administered to effect in horses with chronic obstructive pulmonary disease (COPD) [see comments]. *Equine Veterinary Journal*, **26**, 331–336.
- Gabrielsson, J. & Weiner, D. (2000) *Pharmacokinetic and pharmacodynamic data analysis: Concepts & Application*. Swedish Pharmaceutical Press, Stockholm. 314–315.
- Guan, F., Ubob, C.E., Soma, L.R., Luo, Y., Li, R., Birks, E.K., Teleis, D., Rudy, J.A. & Tsang, D.S. (2002) Quantification of clenbuterol in equine plasma, urine and tissue by liquid chromatography coupled on-line with quadrupole time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, **16**, 1642–1651.
- Hagedorn, H.W., Zuck, S. & Schulz, R. (1995) Detection of clenbuterol (Ventipulmin) in the horse. *Zentralblatt für Veterinärmedizin – Reihe A*, **42**, 209–219.
- Harkins, J.D., Robinson, N.E., Woods, W.E., Lehner, A.F., Smith, M.D., Gates, R.S., Fisher, M. & Tobin, T. (2000) Intratracheal clenbuterol in the horse: its pharmacological efficacy and analytical detection. *Journal of Veterinary Pharmacology and Therapeutics*, **23**, 251–260.
- Johnston A.M. & Dunsire J.P. (1996) Plasma kinetics, metabolism, excretion and residue kinetics of [¹⁴C]-N-AB 365 Cl following oral administration to the horse. Submitted to World Health Organization by Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany. Cited: Woodward, K.N. Toxicological evaluation of certain veterinary drug residues in food. *WHO Food Additives Series*, **38**, 35–43.
- Kallings, P., Ingvast-Larsson, C., Persson, S., Appelgren, L.E., Forster, H.J. & Rominger, K.L. (1991) Clenbuterol plasma concentrations after repeated oral administration and its effects on cardio-respiratory and blood lactate responses to exercise in healthy Standardbred horses. *Journal of Veterinary Pharmacology & Therapeutics*, **14**, 243–249.
- Kleemann, R., Goossens, L., Reder, E. & Quirke, J.F. (1999) Depletion kinetics of clenbuterol hydrochloride in competition horses. *Equine Veterinary Journal*, **31**, 339–341.
- Kopitar, Z. & Zimmer, A. (1976a) [Comparison of the pharmacokinetic profiles of clenbuterol in rat and dog during toxicological studies (author's transl)]. *Arzneimittel-Forschung*, **26**, 1450–1455.

- Kopitar, Z. & Zimmer, A. (1976b) [Pharmacokinetics and metabolite pattern of clenbuterol in the rat]. *Arzneimittel-Forschung*, **26**, 1435–1441.
- Lehner, A.F., Harkins, J.D., Karpiesiuk, W., Woods, W.E., Robinson, N.E., Dirikolu, L., Fisher, M. & Tobin, T. (2001) Clenbuterol in the horse: confirmation and quantitation of serum clenbuterol by LC-MS-MS after oral and intratracheal administration. *Journal of Analytical Toxicology*, **25**, 280–287.
- Mair, T.S. (1996) Obstructive pulmonary disease in 18 horses at summer pasture. *Veterinary Record*, **138**, 89–91.
- Morgan, D.J. (1990) Clinical pharmacokinetics of beta-agonists. *Clinical Pharmacokinetics*, **18**, 270–294.
- Petkova, M. & Stanchev, H. (1996) Study on the effect of clenbuterol on fattening calves. *Zhivotnov'Dni Nauki*, **33**, 44–48.
- Popa, V. (1986) Respiratory pharmacology. Beta-adrenergic drugs. *Clinics in Chest Medicine*, **7**, 313–329.
- Powers, J. (1990) Statistical analysis of pharmacokinetic data. *Journal of Veterinary Pharmacology & Therapeutics*, **13**, 113–120.
- Sauer, M.J., Pickett, R.J., Limer, S. & Dixon, S.N. (1995) Distribution and elimination of clenbuterol in tissues and fluids of calves following prolonged oral administration at a growth-promoting dose. *Journal of Veterinary Pharmacology & Therapeutics*, **18**, 81–86.
- Shapland, J.E., Garner, H.E. & Hatfield, D.G. (1981) Cardiopulmonary effects of clenbuterol in the horse. *Journal of Veterinary Pharmacology & Therapeutics*, **4**, 43–50.
- Smith, D.J. & Paulson, G.D. (1997) Distribution, elimination, and residues of [¹⁴C]clenbuterol HCl in Holstein calves. *Journal of Animal Science*, **75**, 454–461.
- Stefanovski, D., Moate, P.J. & Boston, C. (2003) WinSAAM: a windows based compartmental modeling system. *Journal of Metabolism*, **52**, 1153–1166.
- Toutain, P.L. & Lassourd, V. (2002) Pharmacokinetic/pharmacodynamic approach to assess irrelevant plasma or urine drug concentrations in postcompetition samples for drug control in the horse [comment]. *Equine Veterinary Journal*, **34**, 242–249.
- Wastney, M.E., Patterson, B.H., Linares, O.A., Greif, P.C. & Boston, R.C. (1999) *Investigating Biological Systems Using Modeling: Strategies and Software*. Academic Press, New York.
- Weast, R.C. (1973) *Handbook of Chemistry and Physics*. CRC Press, Cleveland, OH, D-126.
- Yamamoto, I., Iwata, K. & Nakashima, M. (1985) Pharmacokinetics of plasma and urine clenbuterol in man, rat, and rabbit. *Journal of Pharmacokinetics-Dynamics*, **8**, 385–391.
- Zalko, D., Bories, G. & Tulliez, J. (1998a) Metabolic fate of clenbuterol in calves. *Journal of Agricultural Food and Chemistry*, **46**, 1935–1943.
- Zalko, D., Debrauwer, L., Bories, G. & Tulliez, J. (1998b) Metabolism of clenbuterol in rats. *Drug Metabolism & Disposition*, **26**, 891–899.
- Zalko, D., Perdu-Durand, E., Debrauwer, L., Bec-Ferte, M.P. & Tulliez, J. (1998c) Comparative metabolism of clenbuterol by rat and bovine liver microsomes and slices. *Drug Metabolism & Disposition*, **26**, 28–35.
- Zimmer, A. (1976a) [Pharmacokinetics and metabolite pattern of clenbuterol in rabbit and dog (author's transl)]. *Arzneimittel-Forschung*, **26**, 1442–1445.
- Zimmer, A. (1976b) [Single and multiple applications and metabolite pattern of clenbuterol in man (author's transl)]. *Arzneimittel-Forschung*, **26**, 1446–1450.

Exhibit 22



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California takes its drug testing seriously

Posted: Friday, February 26, 2010

DEL MAR -- Just how small is 25 picograms? If you wrote out the number, you'd have to put 10 zeros between the decimal point and the number 25. That's how infinitesimal an amount the California Horse Racing Board can test for in racehorses, which is why California's drug-testing program is the envy of the nation.

"California takes drug testing more seriously than anyplace in the country," said Jerry Brown, creator of the Thoro-Graph sheets used by both bettors and horsemen. "California now has the cleanest racing in the country."

Dr. Rick Arthur, California's equine medical director, notes that California's drug-testing requirements in many ways are stricter than those for any human athletic endeavor. Most Little Leaguers and Olympic competitors couldn't pass the state's post-race testing. Yet day in and day out, Thoroughbred racehorses successfully pass this rigorous testing procedure.

"We're almost at the point where if you had a cup of coffee at Starbucks three days before the race, you could test positive for caffeine at a level that would be a violation in horse racing," said Arthur. "I can't get a cup of coffee even from Starbucks to keep me awake for three days. In the Olympics, the threshold level for caffeine is 12,000 nanograms (parts per billion). In the '90s California horse racing called a positive at two nanograms."

"Most people in the car next to you driving down the road could not pass a post-race drug test. Most people sitting next to you in church could not pass a post-race drug test."

The technology has improved to the extent that California's Kenneth L. Maddy Equine Analytical Laboratory, which tests all of California's samples, can find up to 800 drugs. Arthur says that the lab is adding new substances it can test for all the time.

Why do the tests call positives at levels that are so far below what could possibly influence a horse's performance?

"We err on the side of caution," explained Arthur. "If anything, we over-regulate horse racing for two reasons—1) to protect the integrity of the competition and 2) to protect the welfare of the horse and jockey."

Brown said that California made great strides a couple of years ago when the state began testing for alkalinizing agents (in racing commonly called milkshakes) and changed its testing for clenbuterol from a urine test to a more accurate blood test.

"That has now made racing there the most drug-free and formful," said Brown. "I don't mean formful in the sense of favorites winning, but you don't have to handicap the trainers as much. You can handicap the horses. What you don't see are the extreme form reversals when horses enter or leave certain trainers' barns that you do in the rest of the country. You have no idea how significant that is to bettors in deciding where to bet."

California's landmark decision to test for milkshakes—the process of administering sodium bicarbonate (TCO2) in the hopes that the horse will run faster because his muscles don't build up as much lactic acid—virtually eliminated the problem.

"Our last violation was in August of 2008," said Arthur. "In the last two years in California, we've had only two violations out of over 50,000 samples. That's a pretty amazing success rate."

Clenbuterol is a medication that horsemen use to help horses breathe better. While veterinarians can legally administer clenbuterol to horses in training, it must be withdrawn 72 hours prior to racing. Only that trace amount of 25 picograms per milliliter (parts per trillion) of blood is allowed in a post-race test, an amount deemed far too small to have any effect on a horse's racing performance.

Positives are called both for illegal substances with no legitimate use in the horse and for overages of otherwise legal therapeutic medications. By far the most positives occur in the latter instance, usually when a horse received a medication too close to race time for the drug to clear the system.

"Most drug violations are mistakes," Arthur said, "inadvertent administrations of legitimate medications that were prescribed to the horse with no intent to influence the outcome of a race. The groom, assistant trainer, or trainer may have forgotten that the horse was entered, or the medication was accidentally given to the wrong horse."

"Drugs that one would even suspect are being administered to try to beat the system are few and far between. They are very rare."

Contamination can also cause positive tests. Several years ago, horses in the care of several prominent trainers tested positive for scopolamine. The culprit turned out to be jimsonweed, a poisonous plant containing scopolamine, which had contaminated



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the baled straw bought for the horses' stall bedding.

Human drugs of abuse are another source of violations. If a backstretch worker has a drug problem, traces of the drug can get into a horse's system just through the employee handling the horse.

California officials don't only rely on post-race drug tests to keep horse racing clean. The CHRB now conducts out-of-competition testing, where a horse is tested after training, but well before it is scheduled to race. The reason for that is that some substances, primarily blood-doping agents, can cause an effect over long periods of time but wouldn't be detectable in the standard post-race sample. Human sports testing has the same problem. The California program is similar to out-of-competition testing in the Olympics.

Arthur said that the CHRB has never had a positive through this testing program. The out-of-competition testing is acting as a deterrent.

"Trainers don't want positives," Arthur said. "You get the purse taken away, you get a fine, you get a suspension, your reputation is tarnished. Nothing good comes out of a positive for anybody. Most people try to play by the rules."

California's stringent drug-testing policies reassure trainers that they have a level playing field.

Though sometimes the rules can seem complicated, Arthur tries to be as available as possible to answer questions. A typical conversation might be, "My horse was mistakenly treated five days before the race. Will I be okay?" Or the racing office may write a last-minute race that suits a horse who has been receiving a medication, and the trainer asks Arthur if the medication will have time to clear. Perhaps the horse needed to be tranquilized for special shoes such as glue-ons, and the trainer wants to be sure the tranquilizer was given far enough in advance of a race to be out of the horse's system.

California has also led the way in testing for anabolic steroids. In the summer of 2008, a ban on most major anabolic steroids was phased in, with the CHRB giving trainers notice of when positives would begin to be called.

"Once anabolic steroids were re-classified as Class 3 drugs in September of 2008, we have had no positives," Arthur said. "The program has gone much better than expected."

Another tribute to California's strict testing has come as the national Racing and Medication Testing Consortium adopts standards first implemented in California.

Despite such rigorous testing, rumors persist of illegal cocktails of nefarious drugs for purchase that will make a horse run faster.

"The RMTC has looked at some of these," Arthur said. "We once purchased a special cocktail that cost \$2,000 an ounce that ended up to be water and food coloring. A lot of this stuff is pure nonsense, but we take everything we hear seriously."

"Dr. Scott Stanley is the chief chemist at the Maddy Lab. We are continually looking at our program. We want to know if there are any gaps in our drug coverage before anyone else does. Both Dr. Stanley and I are well connected with drug testing labs in the U.S. and around the world. We have a very good idea what we need to be watching for."

"And we freeze samples in California. We have nearly two years of samples in the freezer. If someone takes a shot at our program, they have a long time to worry about their test clearing."

It's easier and cheaper just to play by the rules.

Clenbuterol

Lynn Hovda

Exhibit 23

From: scot waterman [swaterman@rmtcnet.com]
Sent: Monday, August 30, 2010 9:19 AM
To: Lynn.Hovda@state.mn.us
Subject: RE: Methocarbamol detection limits and other questions

Lynn-

- ✱ 1. Methocarbamol...we used a 15 mg/kg IV dose. We will likely issue a recommendation on this one in the next two months. Based on the preliminary data I have seen, concentrations at 24 hours are below 15 ng/ml in plasma with most horses below 5 ng/ml. We will probably do a small number of horses with an oral dose just because we know that is being used as well but the initial recommendation will be based on the IV dose only.
- ✱ 2. Clenbuterol...pretty simple; 25 pg/ml in blood regulates the 1.6 oral dose with a 72-hour withdrawal and 10 pg/ml in blood regulates a 96-hour withdrawal. We are still struggling with a consensus on 72 vs 96.
3. Corticosteroids...no clue where the AAEP recommendation came from. In fact, I thought their recommendation was five days. Anyway, I don't know that anyone knows what the right number of days is. But, what I can tell you is that whatever recommendation comes down, the threshold will probably be in the low picograms/ml in plasma. And by low I mean somewhere between 5-50 pg/ml depending on the corticosteroid involved. The desire will likely be to regulate IA use 5-15 days out...not sure where it lands.
4. Naproxen...we won't do anything with this drug unless an equine version comes out again. Warn people to back off because it hangs around a very long time. 5-7 days at least.

Probably not making it to AAEP this year. It is too difficult to do both that and the Racing Symposium when AAEP is on the east coast.

Let me know if you have any other questions.

Scot

From: Lynn Hovda [mailto:Lynn.Hovda@state.mn.us]
Sent: Sunday, August 29, 2010 5:50 PM
To: swaterman@rmtcnet.com
Subject: Methocarbamol detection limits and other questions

Scot:

Can you tell me where the RMTC is in terms of detection limits for the 8 drugs you have been working on. I am especially interested in methocarbamol as it has been a persistent problem for us at both racetracks this year. Actually, our problem children are clenbuterol, dexamethasone, methocarbamol, methylprednisolone, naproxen, and triamcinolone.

Corticosteroids have been a very sticky issue for us this year. Any wisdom you can share on where the RMTC is on corticosteroids would be much appreciated. Any wisdom on where the AAEP recommendation for 10 days came from would also be appreciated.

See you in December?

Lynn

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Pharmacokinetics and metabolism of dantrolene in horses

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Dantrolene is a skeletal muscle relaxant used commonly in performance horses to prevent exertional rhabdomyolysis. The goal of the study reported here was to begin to characterize cytochrome P450-mediated metabolism of dantrolene in the horse and describe the pharmacokinetics of the compound, formulated as a capsule or a compounded paste formulation, following oral administration. Dantrolene is rapidly metabolized to 5-hydroxydantrolene both *in vivo* and *in vitro*. Preliminary work with equine liver microsomes suggest that two enzymes are responsible for the metabolism of dantrolene, as evidenced by two distinct K_m values, one at high and one at low substrate concentrations. For the pharmacokinetic portion of the study, a randomized, balanced 2-way crossover design was employed wherein eight healthy horses received a single oral dose of either capsules or paste followed by a 4 week washout period prior to administration of the second formulation to the same horse. Blood samples were collected at time 0 (prior to drug administration) and at various times up to 96 h postdrug administration. Plasma samples were analyzed using liquid chromatography-mass spectrometry and data analyzed using both noncompartmental and compartmental analysis. Peak plasma concentrations were 28.9 ± 21.6 and 37.8 ± 12.8 ng/mL for capsules and paste, respectively and occurred at 3.8 h for both formulations. Dantrolene and its major metabolite were both below the limit of detection in both plasma and urine by 168 h postadministration.

(Paper received 2 March 2010; accepted for publication 4 June 2010)

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INTRODUCTION

Dantrolene is classified as a Class 4 (Penalty Class C) foreign substance by the Association of Racing Commissioners International (ARCI) and while its use prior to racing is not permitted, it is commonly used therapeutically before strenuous training to prevent exertional rhabdomyolysis (tying up). It is a skeletal muscle relaxant that acts by suppressing calcium release, subsequently interfering with excitation-contraction coupling in the muscle fiber. Its effectiveness in racehorses undergoing strenuous exercise is for the most part anecdotal, as there are no studies demonstrating the efficacy of this drug for the treatment of exertional rhabdomyolysis. There are a few published reports describing its utility in decreasing creatinine kinase (CK), a marker of muscle damage. McKenzie *et al.* (2004) administered an oral dose of either 4, 6 or 8 mg/kg of dantrolene to horses with recurrent exertional rhabdomyolysis (RER) 90 min prior to exercise and were able to demonstrate significant decreases in CK

as well as alleviation of the clinical signs associated with tying up. Similarly, Edwards *et al.* (2003) were able to demonstrate a significant difference in pre- vs. posttrace CK levels, between horses with exertional rhabdomyolysis and those without, following dose of 800 mg of dantrolene orally, 1 h prior to exercise.

To the authors' knowledge, there is only one previous report describing the pharmacokinetics of dantrolene in horses (Court *et al.*, 1987). In that study, two ponies and four horses received dantrolene, both intravenously and intragastrically with blood and urine samples being collected at various times postadministration. Following intravenous administration, the parent compound appears to be rapidly distributed and eliminated from the body, with an elimination half-life of 129 ± 8 min and a whole body clearance of 4.16 ± 0.52 mL/min/kg. The authors also concluded that dantrolene is quickly absorbed following oral administration and similar to intravenous administration, rapidly eliminated. In other species, dantrolene is rapidly

metabolized and eliminated primarily as a hydroxylated compound (Wuis *et al.*, 1990; Jayyosi *et al.*, 1993; Krause *et al.*, 2004); however, whether this is the same for the horse remains to be determined, as there are no specific reports in the literature regarding the metabolism of dantrolene.

In the United States, there are currently no Food and Drug Administration (FDA) products labeled for veterinary use and as such, the only permitted product is the human-approved product, Dantrium (Proctor & Gamble Pharmaceuticals, Cincinnati, OH, USA). Dantrium is available in both an injectable formulation for intravenous administration as well as capsules for oral administration. While it is often cost prohibitive to use the injectable formulation in veterinary medicine, especially in animals as large as the horse, the use of capsules is common. Frequently, capsules will be dissolved in water and administered orally using a dosing syringe. Another common practice is to administer paste formulations that can be easily obtained from veterinary compounding pharmacies. Under the Animal Medicinal Drug Use and Clarification Act (AMDUCA), compounding from bulk drug would be illegal and as such, compounding pharmacies should be compounding from FDA-approved products, such as Dantrium. However, because of limited resources, regulation of veterinary compounded formulations by the FDA-CVM is often times not a priority, and this should be taken into consideration when administering these products. In the study reported here, it is unknown whether the compounded product was formulated from bulk drug or an approved product.

The purpose of the study reported here was twofold. First, we sought to begin to characterize cytochrome P450-mediated metabolism of dantrolene in the horse, utilizing equine liver microsomes. The second goal of this study was to describe plasma concentrations with respect to time following oral administration of dantrolene as a means of recommending an appropriate withdrawal time prior to competition. In the previous report describing the pharmacokinetics of dantrolene in the horse, the compound was administered intragastrically. However, on the racetrack, it is much more common to administer dantrolene orally via a dosing syringe and as such, we sought to mimic these conditions as closely as possible. Additionally, we administered a compounded paste formulation of dantrolene so as to describe the relative oral bioavailability to that of the FDA-approved Dantrium capsules, when administered as a suspension dissolved in water. Plasma concentrations of the primary metabolite, as determined from *in vitro* incubations, were also measured in the *in vivo* drug administration study and full pharmacokinetic analysis of the metabolite performed.

MATERIALS AND METHODS

In vitro metabolism studies

Preparation of equine liver microsomes. Liver samples were collected from three adult thoroughbred horses (two females and one castrated male). The samples were collected from horses euthanized for other studies previously approved by the Institu-

tional Animal Care and Use Committee of the University of California at Davis. All horses were determined to be healthy by physical exam prior to euthanasia. Therapeutic medications that had the potential to affect the expression of metabolic enzymes were noted. Liver samples (100–200 g) were collected within 20 min of the horses being euthanized and placed in ice cold 0.9% saline for transport back to the laboratory.

Liver samples were rinsed and flushed with ice cold 0.9% saline using visible arteries, veins or ducts. Samples were blotted dry, weighed, chopped into small pieces and placed in an ice cold blender. Liver samples were homogenized by pulse blending several times, followed by continuous blending on the lowest setting for approximately 20 sec, in three volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM KCl and 2 mM EDTA. The mixture was further homogenized with a motor-driven Teflon/glass tissue grinder. Samples were then subject to a series of differential centrifugation steps. The mixture was first centrifuged at 9000 *g* for 20 min followed by further centrifugation at 100 000 *g* for 60 min, following removal of the supernatant and resuspension in the Tris-HCl buffer described earlier. This centrifugation step was repeated one additional time and the final pellet resuspended by hand, using a teflon/glass homogenizer, in 100 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 20% glycerol and 1 mM dithiothreitol. Protein content was determined using the bicinchoninic acid assay (BCA). P450 levels were determined by obtaining the difference spectra of sodium dithionite-reduced vs. CO-bubbled samples at 500–400 nm according to the methods of Omura and Sato (1964). Microsomes were stored at -80° until used.

Equine liver microsomal incubations. Microsomes were not pooled for the incubation reactions, rather incubations for each horse were run in separate test tubes. Microsomal incubations consisted of 1 mg/mL protein in 100 mM potassium phosphate buffer (pH 7.4). All reactions (liver microsomes + varying concentrations of dantrolene) were incubated at 37 °C for 2 min prior to initiation of the reaction by the addition of 1 mM NADPH. Reactions were allowed to proceed for 5 min and were terminated by the addition of 250 μ L of acetonitrile (ACN) containing nitrofurantoin as the internal standard (IS). Dantrolene hydroxylase activity was measured under linear conditions using a varying number of substrate concentrations ranging from 0 to 800 μ M. Velocity of the reaction was calculated as pmol product/min/pmol of CYP450. The data was evaluated by plotting velocity (*V*) vs. *V*/[Substrate] (Eadie Hofstee plot), and the intercepts calculated by linear regression analysis. Apparent K_m and V_{max} values were calculated from the Eadie Hofstee plot.

In vivo pharmacokinetics

Animals. Eight healthy unmedicated adult horses including seven Thoroughbreds and one Standardbred (four geldings and four mares with a mean \pm SD weight of 582.8 \pm 51.7 kg and an age of 10 \pm 4 years) were studied. Food was withheld for 12 h prior to and for 12 h following drug administration. Water was available *ad libitum* throughout the study. Before beginning the

study, horses were determined healthy and free of disease by physical examination, complete blood count (CBC) and a serum biochemistry panel that included aspartate aminotransferase (AST), creatinine phosphokinase (CK), alkaline phosphatase (ALP), total bilirubin, sorbital dehydrogenase (SDH), blood urea nitrogen (BUN) and creatinine. Blood analyses were performed by the Clinical Pathology Laboratory of the William R. Pritchard Veterinary Medical Teaching Hospital of the University of California, Davis, using standard protocols. Horses did not receive any other medications for at least 2 weeks prior to commencement of this study. This study was approved by the Institutional Animal Care and Use Committee of the University of California at Davis.

Instrumentation and drug administration. This study was conducted in a randomized, balanced 2-way crossover design, wherein four horses received the paste formulation and four horses received capsules. Following a 4 week washout period, the groups were reversed and horses received the second formulation. Prior to drug administration, a 14-gauge intravenous catheter was percutaneously placed in one external jugular vein for sample collection. Each horse was weighed preceding drug administration. The dose for both formulations was 500 mg.

Dantrolene capsules (Dantrium; Proctor & Gamble Pharmaceuticals) were dissolved in water in a dosing syringe immediately prior to administration and delivered directly into the posterior portion of the oral cavity. The paste formulation was purchased from a large commercial compounding pharmacy and was administered as purchased. As this was a compounded formulation, the actual dantrolene concentration of the paste product was measured, as described in the Sample Analysis section.

Sample collection. Blood samples were collected at time 0 (prior to drug administration) and at 15, 30, and 45 min, and 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 18, 24, 36, 48, 72 and 96 h postdrug administration. Catheters were removed following collection of the 8-h sample, and the remaining samples were collected by direct venipuncture. Blood samples were collected into EDTA blood tubes (Kendall/Tyco Healthcare, Mansfield, MA, USA) and were centrifuged at 3000 *g* for 10 min. Plasma was immediately transferred into storage cryovials (Phenix Research Products, Chandler, NC, USA) and stored at -20 °C until analysis. Urine samples were collected via a 30 french rubber urinary catheter (Kendall/Tyco Healthcare) from three horses (two mares and one gelding) prior to and at 96 and 168 h postdantrolene administration. Urine was collected from the same three horses for both the capsule and paste formulations. To ensure the safety of both horses and handlers, urine samples were only collected from horses previously catheterized. All samples were stored at -20 °C (approximately 2 weeks) until analysis.

Sample analysis. Dantrolene and nitrofurazone were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-hydroxydantrolene

was purchased from Toronto Research Chemicals (North York, ON, Canada). Standards were purchased as powders and 1 mg/mL solutions were prepared prior to analysis. Nitrofurazone was utilized as the IS. High performance liquid chromatography (HPLC) grade ACN, water, methyl *tert*-butyl ether (MTBE), ethyl acetate and methanol were purchased from Burdick & Jackson (Muskegon, MI). Formic acid was purchased from Acros Organics (Morris Plains, NJ, USA).

The purity and strength of dantrolene paste was determined by extracting 1 mL aliquots ($n = 5$) of the compounded product and comparing concentrations to a dantrolene reference standard. Dantrolene was extracted from paste using a modified extraction procedure outlined by Katogi *et al.* Briefly, 1 mL aliquots using the measuring device provided on the products syringe and diluted in 9 mL of water. The solution was rotor-mixed for 5 min followed by sonication at 60 °C for 5 min. The solution was subsequently diluted 1:10 once more in water. A 0.5-mL aliquot of the 1:100 diluted paste was placed into a tube containing an IS and 2 mL of 0.1 M Acetic Acid (pH 4.0) was added and tube shaken for 1 min. Dantrolene was extracted by adding 5 mL of ethyl acetate to the tube containing the diluted dantrolene paste and rotor-mixed for 10 min. The tube was centrifuged at 1300 *g* for 10 min and organic layer decanted and dried under N₂ at 50 °C using a Zymark Turbovap concentrator (Hopkinton, MA, USA). Extracts were reconstituted in 200 µL of ACN followed by a 5 µL injection onto the liquid chromatography-mass spectrometry/MS system to determine concentration of dantrolene in the compounded product. Dantrolene reference standards were processed in a similar fashion and analyte peak area to IS area ratios compared between the standard and the dantrolene paste.

A liquid-liquid extraction method was employed to separate dantrolene, 5-hydroxydantrolene and the IS from equine plasma. Briefly, 1 mL aliquots of plasma were added to tubes containing 100 ng the IS and mixed for 30 sec. Analytes were extracted following the addition of 5 mL of MTBE and rotor-mixed for 5 min. The organic layer was separated and evaporated to dryness under N₂ on a Zymark Turbovap concentrator at 40 °C. Samples were redissolved in 150 µL of 10:90 ACN:water with 0.2% formic acid.

Calibrators and quality control (QC) samples were prepared as described earlier. Drug-free equine plasma was used in the generation of calibrators, negative control and QC samples. Calibrators and QC samples were prepared by spiking drug-free equine plasma with working standards of 5-hydroxy-dantrolene and dantrolene. Final concentrations for calibrators were 0.1, 1, 5, 10, 25, 50, 100, 250 and 500 ng/mL. Calibrators were run at the beginning and end of each sample set. QC samples ($n = 6$ per level) were used to determine accuracy and precision at 10 and 100 ng/mL in blank plasma.

Chromatographic separations were performed on an Agilent 1100 Series HPLC system (Palo Alto, CA, USA). Samples were introduced by a 40 µL injection and separated on an ACE 3 C18 (2.1 × 100 mm, 3 µm particle size) column (Mac-mod Analytical Inc, Chadds Ford, PA, USA) maintained at 30.0 °C by a column heater. Solvents A and B were water with 0.2% formic

acid and ACN with 0.2% formic acid, respectively. Total run-time for each sample was 10.00 min. Analytes were separated over a 10-min method using a reverse phase gradient in the following steps: (i) 0–0.3 min at 0.35 mL/min of a 95:5 ratio of mobile phases A:B, Fig. 1 (ii) 0.3–5.0 min at 0.35 mL/min of 10:90 A:B, (iii) 5.0–6.0 min at 0.35 mL/min holding of 10:90 A:B, (iv) 6.0–8.0 min at 0.35 mL/min of 95:5 A:B, (v) 8–10 min at 0.45 mL/min of 95:5 A:B. Retention times were reproducible over the study period with dantrolene, 5-hydroxydantrolene and the nitrofurazone eluting at 7.12, 7.04 and 5.54 min, respectively.

Following chromatographic separation and introduction by negative mode electrospray ionization analytes were quantitated on a Thermo LTQ XL ion-trap mass spectrometer (MS) (San Jose, CA, USA). Data acquisition was performed using the Thermo Xcalibur 2.0 software (San Jose, CA, USA) and quantitation performed using Quan Browser. The following $[M-H]^-$ pseudo-molecular ions were monitored: Dantrolene (313.0 m/z), 5-hydroxydantrolene (329.0 m/z) and nitrofurazone (197.0 m/z). MS/MS of the analytes was performed using a isolation width of 1.5, 1.5 and 2.0 and collision energy of 30, 30, 32 for dantrolene, 5-hydroxydantrolene and nitrofurazone, respectively. The following product ions were used for the quantification of dantrolene (228.0, 270.1 m/z), 5-hydroxydantrolene (257.0, 286.2 m/z) and nitrofurazone (124.0, 150.0, 167.0 m/z).

Calibration curves for both dantrolene and 5-hydroxydantrolene were generated by weighted ($1/X$) quadratic regression analysis using the ratio of analyte peak area to the IS peak area with correlation coefficients (r^2) >0.99 . The experimentally determined limit of quantitation was 0.1 ng/mL for both dantrolene and 5-hydroxydantrolene. Inter- and intraday ($n = 2$ days) precision (%CV) was assessed at 10 and 100 ng/mL for both analytes.

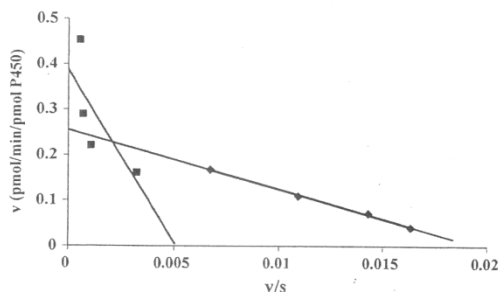


Fig. 1. Eadie Hofstee plot for the determination of the apparent $K_{m\max}$ and V_{\max} values for dantrolene metabolism by equine liver microsomes. Incubations contained 1 mg/mL liver microsomes, NADPH and dantrolene (0, 2.5, 5, 10, 25, 50, 200, 400 and 800 μM) and were incubated for 5 min at 37 °C. Values are the mean from data obtained from three different horses, two separate incubations per horse at each substrate concentration.

Pharmacokinetic calculations. Nonlinear least square regression was performed on plasma dantrolene and hydroxy-dantrolene concentrations using commercially available software (WinNonlin Version 5.2; Pharsight, Cary, NC, USA). Parent data was analyzed using noncompartmental analysis (NCA) and compartmental modeling based on the Gauss–Newton (Levenberg and Hartley) method. Only NCA was used to analyze metabolite data. Data points included in analysis were plasma concentration values until drug was no longer detected (36 h postadministration). Coefficient of variation, Akaike information criterion (AIC) and visual inspection of the residual plots were used to determine the goodness of fit of models and the appropriate weighting scheme for the individual horse data. Values from all eight horses are reported as mean \pm SD. Statistical analyses were performed to assess significant differences in pharmacokinetic parameters between the capsule and paste formulations for both the parent compound and the 5-hydroxydantrolene metabolite. All data were analyzed using a paired t -test based on the differences between the two parameters; a paired t -test based on the differences between log-transformed versions of the two parameters and a nonparametric (Wilcoxon signed rank) test. Significance was set at $P < 0.05$.

RESULTS

Microsomal incubations for dantrolene metabolism were performed for 5 min using substrate concentrations ranging from 0 to 1800 μM . The data was evaluated by plotting velocity (V) vs. $V/[\text{Substrate}]$ (Eadie Hofstee plot), and the intercepts were calculated by linear regression analysis. The kinetics of dantrolene metabolism by equine liver microsomes was found to consist of two discrete phases, as evidenced by two distinct slopes (Fig. 1). Both a low and high apparent K_m and V_{\max} value was calculated for the 5-hydroxydantrolene metabolite (Table 1). The apparent K_m value at low dantrolene concentrations is nearly six times lower when compared to that calculated for high substrate concentrations. However, the maximal rates of substrate metabolism in the microsomal incubations were comparable at both low and high substrate concentrations.

The compounded dantrolene paste was found to contain 17.6 ± 1.8 mg/mL, which corresponds to $87.9 \pm 8.9\%$ of the labeled 20 mg/mL concentration. Individual as well as mean

Table 1. Kinetic parameters of dantrolene metabolism by equine liver microsomes

	5-hydroxydantrolene		
	Apparent K_m (μM)	V_{\max} (pmol/min/pmol CYP450)	V_{\max}/K_m
Low [dantrolene]	13.0	0.25	0.02
High [dantrolene]	76.0	0.39	0.005

Values represent the mean from data obtained from three different horses, two separate incubations per horse at each substrate concentration.

dantrolene plasma concentration values for both the capsule and paste formulations are listed in Tables 2 & 3, respectively. Mean dantrolene plasma concentration vs. time curves for paste and oral formulations are compared in Fig. 2. Mean plasma concentration vs. time data for both dantrolene and 5-hydroxydantrolene following oral administration of 500 mg of capsules and paste are depicted in Figs 3 & 4, respectively. Based on the AIC and visual inspection of residual plots, a one compartment model ($C_p = Ae^{-\alpha t}$) gave the best fit to dantrolene concentration data points from each individual animal. Individual as well as mean values (\pm SD) for a number of pharmacokinetic variables following noncompartmental and compartmental analysis of dantrolene are listed in Tables 4 & 5, respectively. 5-hydroxydantrolene plasma concentrations were analyzed using NCA

only (Table 6). Pharmacokinetic parameters for the capsule and paste formulations were found to be comparable with significant differences between the two formulations noted for the area under the moment curve (AUMC) for dantrolene and the area under the curve (AUC) and AUMC for the 5-hydroxydantrolene metabolite. All urine samples were determined to be below the limit of detection for both dantrolene and its metabolite at 168 h postdrug administration.

DISCUSSION

A common practice on the racetrack is to administer dantrolene prior to strenuous exercise to horses to prevent 'tying up.'

Table 2. Dantrolene plasma concentration data (ng/mL) following a single oral administration of 500 mg of Dantrium capsules to horses ($n = 8$)

Horse									
Time (h)	1	2	3	4	5	6	7	8	Mean \pm SD
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
0.25	1.77	0.92	0.17	10.19	0.31	1.24	9.53	0.35	3.06 \pm 4.24
0.5	10.77	9.63	1.23	31.88	2.10	3.69	37.37	1.09	12.22 \pm 14.38
0.75	11.34	12.33	1.77	44.03	2.83	5.0	55.48	2.11	16.86 \pm 20.92
1	6.50	12.71	3.13	51.21	2.55	4.38	53.63	2.45	17.07 \pm 22.08
1.5	8.57	12.57	9.97	43.96	4.85	8.99	70.33	11.63	20.53 \pm 23.87
2	8.86	10.64	7.96	47.99	6.27	14.20	50.14	27.42	21.94 \pm 17.93
2.5	9.43	9.68	9.64	32.43	9.13	23.51	43.50	29.61	20.66 \pm 13.57
3	15.14	13.26	9.46	31.89	7.38	27.36	36.15	27.48	21.04 \pm 10.95
4	17.42	13.14	10.21	38.31	7.47	19.89	32.16	30.29	21.02 \pm 11.35
6	7.21	4.64	9.44	13.84	8.61	15.31	27.58	23.27	13.83 \pm 8.01
8	8.11	6.39	5.09	13.84	11.17	14.84	21.88	20.70	13.30 \pm 5.67
12	4.74	1.66	1.99	4.55	1.74	5.67	5.52	8.78	4.72 \pm 2.28
18	0.74	0.36	1.18	0.72	0.21	1.39	1.27	1.60	1.04 \pm 0.63
24	0.25	0.37	<LOQ	0.17	<LOQ	0.71	0.90	1.13	0.61 \pm 0.43
36	<LOQ	<LOQ	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
48	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Table 3. Dantrolene plasma concentration data (ng/mL) following a single oral administration of 500 mg of dantrolene paste to horses ($n = 8$)

Horse									
Time (h)	1	2	3	4	5	6	7	8	Mean \pm SD
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
0.25	2.72	3.06	3.50	2.61	0.36	0.3	0.15	0.69	1.67 \pm 1.42
0.5	19.27	30.49	11.18	19.47	2.67	2.42	0.30	4.46	11.28 \pm 10.82
0.75	19.36	31.31	11.44	17.93	6.24	4.31	0.49	18.70	13.72 \pm 10.06
1	43.30	34.75	11.46	21.33	8.91	3.95	0.57	23.43	18.46 \pm 15.05
1.5	35.55	48.92	11.53	26.44	16.44	4.32	1.99	34.65	22.48 \pm 16.65
2	27.26	43.95	16.93	30.55	21.64	4.92	3.78	44.40	24.18 \pm 15.56
2.5	22.11	28.40	20.60	51.56	19.02	3.68	7.07	44.34	24.60 \pm 16.6
3	18.86	24.14	26.82	49.54	19.86	3.46	9.53	49.41	25.2 \pm 16.77
4	15.26	27.84	26.71	43.75	15.69	8.04	23.91	33.67	24.36 \pm 11.37
6	10.31	22.96	40.10	34.36	15.28	11.81	25.16	26.25	23.27 \pm 10.56
8	8.32	16.0	37.8	21.55	11.3	22.41	23.13	23.91	20.55 \pm 9.07
12	4.70	5.04	8.4	7.01	4.92	9.57	17.36	10.26	8.41 \pm 4.21
18	0.64	1.31	1.49	0.83	1.01	0.77	4.28	0.74	1.38 \pm 1.21
24	0.25	3.02	0.46	0.32	0.39	0.25	1.15	0.82	0.83 \pm 0.94
36	<LOQ	0.23	<LOD	<LOD	0.12	<LOQ	0.11	0.11	0.14 \pm 0.06
48	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

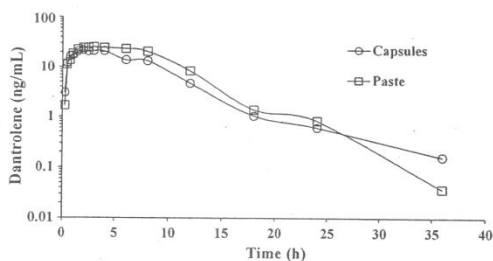


Fig. 2. Average plasma concentration vs. time curves for dantrolene capsules and paste following administration of 500 mg of dantrolene.

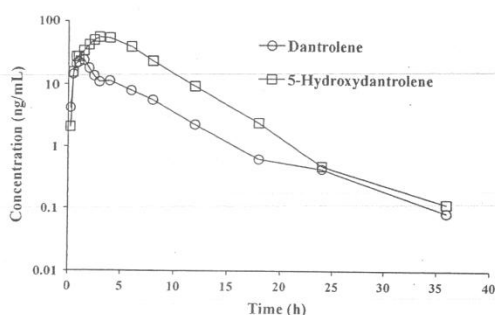


Fig. 3. Average plasma concentration vs. time curves for dantrolene and its major metabolite 5-hydroxydantrolene following administration of 500 mg of dantrolene capsules.

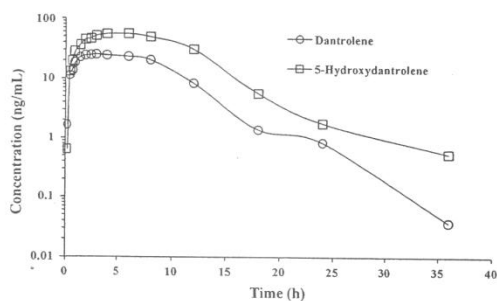


Fig. 4. Average plasma concentration vs. time curves for dantrolene and its major metabolite 5-hydroxydantrolene following administration of 500 mg of dantrolene paste.

However, this drug is also regulated by the ARCI and as such an appropriate withdrawal period must be observed to prevent a positive test. The first goal of the current study was to begin to elucidate the metabolic profile of dantrolene by utilizing both an *in vitro* model of drug metabolism as well as by measuring metabolite concentrations following administration of

dantrolene to horses. The second goal was to describe the pharmacokinetic profile of two formulations of dantrolene when administered orally to horses to recommend an appropriate withdrawal period prior to racing.

In many species, hydroxylation appears to be the primary means of elimination of dantrolene from the body (Wuis *et al.*, 1990; Jayyosi *et al.*, 1993). Based on our analysis of plasma and urine samples, hydroxylation also appears to be the predominant pathway for elimination of dantrolene in the horse. Time to maximal plasma concentrations of both parent compound as well as the 5-hydroxy metabolite was in close agreement; however, the maximal plasma concentrations were strikingly different. Maximal 5-hydroxydantrolene concentrations were nearly twice as high as dantrolene, indicating rapid metabolism of the parent compound. In rats, hydroxylation is dependent upon cytochrome P450 enzymes, with multiple P450s capable of producing the 5-hydroxydantrolene metabolite (Jayyosi *et al.*, 1993). Results from the current study indicate that this may be the case in horses as well, based on results from incubations with equine liver microsomes. Jayyosi *et al.* (1993) determined that in rats, that CYP1A1 was responsible for low K_m hydroxylation while CYP1A2 for high. While further studies are necessary to definitively conclude that P450s are responsible for the metabolism of dantrolene at high and low substrate concentrations in the horse, preliminary results do suggest that metabolism of this compound may be biphasic.

To our knowledge, there is only one previous report describing the pharmacokinetic profile of dantrolene following administration to horses (Court *et al.*, 1987). In that study, investigators administered dantrolene both intravenously and orally. In the current study, the expense associated with administration of the intravenous formulation made its administration cost prohibitive, thereby limiting the number of pharmacokinetic parameters that we were able to calculate. Court and colleagues administered a dose of 4 mg/kg, which based on their reported average horse weight, is equivalent to an average total dose of 1.5 g per horses. This is in stark contrast to the current study in which we administered a total dose of 500 mg to each horse, chosen based on the most commonly used dose on the racetrack. Accordingly, C_{max} values differed dramatically between the two studies, with maximal dantrolene plasma concentrations of 1420 and 28.9 ng/mL, in the Court *et al.* and the current study, respectively. While the large difference in doses between the two studies is the most plausible explanation for differences in C_{max} , another possible explanation for the discrepancy is the method by which the drug was administered. In the study conducted by Court *et al.* (1987), Dantrium capsules were dissolved and the suspension given via a nasogastric tube, delivering the drug directly to the stomach. In the study reported here, a dosing syringe was used and the drug suspension delivered into the back of the oral cavity. A dosing syringe was chosen in this study to mimic, as closely as possible, the conditions on the racetrack. While every attempt was made to ensure that the entire dose was administered, administration via a dosing syringe can be problematic in that all of the dose may not reach the gastrointestinal tract. Another possible explanation for the large

Table 4. Pharmacokinetic parameters of dantrolene following a single oral administration of 500 mg of Dantrium Capsules and Dantrolene Paste to horses ($n = 8$). All values in this table were generated using noncompartmental analysis

Horse	1	2	3	4	5	6	7	8	Mean \pm SD
Weight (kg)	608.7	580.4	564.8	508.3	511.6	620.7	648.5	619.6	582.8 \pm 51.7
T_{max} (h)									
Capsules	4.0	3.0	6.0	1.0	1.5	8.0	3.0	4.0	3.8 \pm 2.3
Paste	1.0	1.5	6.0	2.5	6.0	8.0	2.0	3.0	3.8 \pm 2.6
C_{max} (ng/mL)									
Capsules	17.4	13.3	10.2	51.2	70.3	11.1	27.4	30.3	28.9 \pm 21.6
Paste	43.3	48.9	40.1	51.6	25.2	22.4	21.6	49.4	37.8 \pm 12.8
T_{min} (h)									
Capsules	36	36	24	24	36	24	36	36	31.5 \pm 6.2
Paste	24	36	24	24	36	24	36	36	30.0 \pm 6.4
AUC (h·ng/mL)									
Capsules	129.6	99.6	132.9	282.0	368.7	90.8	198.2	277.0	197.3 \pm 101.7
Paste	180.9	306.8	343.2	350.7	293.6	169.3	172.7	352.6	271.2 \pm 82.9
AUMC (h ² ·ng/mL)*									
Capsules	928.2	604.7	1349.8	1333.3	2043.6	609.0	1503.3	2122.2	1311.8 \pm 582.0
Paste	963.9	2232.5	2414.6	2000.0	2834.7	1449.3	1200.9	2260.7	1919.6 \pm 649.6
MRT (h)									
Capsules	7.1	6.1	10.2	4.7	5.5	6.7	7.6	7.7	7.0 \pm 1.6
Paste	5.3	7.3	7.0	5.7	9.7	8.6	7.0	6.4	7.1 \pm 1.4
λ (per h)									
Capsules	0.16	0.17	0.13	0.28	0.18	0.27	0.12	0.17	0.19 \pm 0.06
Paste	0.21	0.15	0.26	0.26	0.20	0.30	0.17	0.19	0.22 \pm 0.05
$t_{1/2\lambda}$ (h)									
Capsules	4.3	4.2	5.4	2.5	3.8	2.5	5.6	4.2	4.0 \pm 1.1
Paste	3.2	4.7	2.6	2.7	3.4	2.3	4.2	3.6	3.4 \pm 0.8

*denotes a significant difference between paste and capsule formulations. AUC, area under the curve; AUMC, area under the moment curve.

Table 5. Pharmacokinetic parameters of 5-hydroxydantrolene following a single oral administration of 500 mg of Dantrium Capsules and Dantrolene Paste to horses ($n = 8$). All values in this table were generated using compartmental analysis

Horse	1	2	3	4	5	6	7	8	Mean \pm SD
Weight (kg)	608.7	580.4	564.8	508.3	511.6	620.7	648.5	619.6	582.8 \pm 51.7
AUC (h·ng/mL)									
Capsules	131.1	100.5	135.8	269.8	347.6	108.2	212.7	307.3	201.6 \pm 96.7
Paste	181.5	311.0	349.3	348.4	175.5	168.9	296.3	355.9	273.3 \pm 83.7
K01 (per h)									
Capsules	0.64	1.38	0.20	1.54	1.63	0.22	0.25	0.23	0.76 \pm 0.64
Paste	1.47	1.40	0.23	0.32	0.26	0.18	0.14	0.28	0.54 \pm 0.56
K10 (per h)									
Capsules	0.15	0.17	0.19	0.24	0.22	0.21	0.25	0.23	0.21 \pm 0.03
Paste	0.23	0.17	0.22	0.32	0.29	0.15	0.14	0.33	0.23 \pm 0.08
K01 HL(h)									
Capsules	1.1	0.5	3.4	0.5	0.4	3.2	2.8	3.0	1.9 \pm 1.4
Paste	0.5	0.5	3.0	2.2	2.7	3.9	5.0	2.5	2.5 \pm 1.5
K10 HL (h)									
Capsules	4.5	4.0	3.7	2.9	3.2	3.2	2.8	3.0	3.4 \pm 0.6
Paste	3.0	4.1	3.1	2.2	2.4	4.7	5.1	2.1	3.3 \pm 1.2

AUC, area under the curve.

difference in maximal plasma dantrolene concentration in the Court *et al.* (1987) study and this study is differences in analytical methods. In the previous study, Court *et al.* (1987) adopted a previously described method (Hollifield & Conklin, 1973) utilizing a combination of solvent extraction, column chromatography and fluorometry. Relative to the analytical

method described in the current paper, the fluorometric method of detection does not provide the same sensitivity and may not have allowed for differentiation between parent drug (dantrolene) and metabolite (5-hydroxydantrolene) in the sample. In addition to maximal plasma concentrations, the time to maximal concentration (T_{max}) also differed significantly between the

Table 6. Pharmacokinetic parameters of 5-hydroxydantrolene following a single oral administration of 500 mg of Dantrium Capsules and Dantrolene Paste to horses ($n = 8$). All values in this table were generated using non-compartmental analysis

Horse	1	2	3	4	5	6	7	8	Mean \pm SD
Weight (kg)	608.7	580.4	564.8	508.3	511.6	620.7	648.5	619.6	582.8 \pm 51.7
T_{max} (h)									
Capsules	4.0	3.0	6.0	4.0	2.5	6.0	3.0	4.0	4.1 \pm 1.3
Paste	1.0	2.0	6.0	4.0	6.0	8.0	3.0	3.0	4.1 \pm 2.4
C_{max} (ng/mL)									
Capsules	54.4	31.0	35.7	81.0	127.1	34.1	65.2	76.6	63.1 \pm 32.3
Paste	71.9	77.3	95.4	87.9	55.1	55.1	44.9	74.8	70.3 \pm 17.4
T_{min} (h)									
Capsules	36	24	24	24	24	18	24	24	24.8 \pm 5.0
Paste	24	36	24	24	48	48	36	72	39.0 \pm 16.7
AUC (h·ng/mL)*									
Capsules	401.3	194.2	404.2	593.1	709.6	199.5	322.9	525.3	418.8 \pm 183.0
Paste	525.0	666.6	940.5	753.2	719.1	547.4	447.1	791.7	673.8 \pm 161.7
AUMC (h ² ·ng/mL)*									
Capsules	2956.4	1299.5	3663.1	3367.5	3097.9	1004.7	1825.2	3147.4	2545.2 \pm 1014.6
Paste	3292.9	4723.2	7339.1	5051.7	8141.2	5947.7	3720.7	7477.4	5711.7 \pm 1810.7
MRT (h)									
Capsules	7.4	7.0	9.1	5.7	4.4	5.0	5.7	6.0	6.2 \pm 1.5
Paste	6.3	7.1	7.8	6.7	11.3	10.9	8.3	9.4	8.5 \pm 1.9
λ (per h)									
Capsules	0.20	0.17	0.18	0.32	0.31	0.42	0.32	0.31	0.28 \pm 0.09
Paste	0.33	0.20	0.36	0.34	0.13	0.04	0.15	0.10	0.20 \pm 0.12
$t_{1/2\lambda}$ (h)									
Capsules	3.5	4.1	4.0	2.2	2.3	1.6	2.2	2.2	2.8 \pm 0.9
Paste	2.1	3.5	1.9	2.0	5.4	15.8	4.8	8.0	5.4 \pm 4.7

*denotes a significant difference between paste and capsule formulations. AUC, area under the curve; AUMC, area under the moment curve.

previous study (Court *et al.*, 1987) and the current study. In the previous study, maximal plasma concentrations were achieved at 1.7 h in contrast to nearly four hours in the current study. Again, the delay in absorption in this study is most likely attributable to the means by which the drug was administered. Drug was delivered essentially as a bolus by Court *et al.* (1987), while in this study it was delivered as a suspension via an oral dosing syringe. It is also interesting to note, the large variability in maximal and time to maximal plasma concentrations between horses in this study. Possible explanations include differences in absorption or metabolism of the drug or quite simply differences in the amount of drug lost during administration to different horses.

In addition to the FDA-approved capsules, in this study, we administered a paste formulation of dantrolene, readily available from compounding pharmacies and widely used in veterinary medicine. Even though paste formulations of dantrolene are easily obtainable from reputable suppliers, it is important to note that these products, including the one used in this study, are manufactured by veterinary compounding pharmacies and as such are not subject to the same regulations and scrutiny as products that have undergone the FDA approval and manufacturing processes. In this particular study, we chose to analyze the product prior to administration to ensure that the concentration was as listed on the label. The lot number used here was in fact within the concentration requirements set forth by the FDA; however, it is important to note that this applies only to the lot

that was used in this study and does not necessarily hold true for other lots purchased from the same or different manufacturers in the future. For both formulations, a total dose of 500 mg was administered with maximal plasma concentrations of 28.9 and 38 ng/mL for capsules and paste, respectively. Similarly T_{max} values were comparable for the two formulations (Fig. 2). For both formulations, concentrations of the parent compound were below the limit of detection in the central compartment by 36 h postdrug administration. In addition to blood, in the current study, we chose to collect urine samples from a subset of the study horses following the last blood collection. This was carried out to ensure that in addition to blood, that urine dantrolene levels were below the limit of quantitation, as it is common practice for many drug testing laboratories to analyze urine samples for evidence of dantrolene administration. In all cases, the drug and its metabolite was below the limit of detection by 168 h postdrug administration for both capsules and paste formulations. Once again, it should be noted that the paste formulation was a compounded product and the values reported here only apply to the lot used in this study. Other lot numbers of the compounded paste may or may not produce comparable results to those seen with the lot tested here or the FDA-approved product (Dantrium capsules).

In the study reported here, we have determined a number of pharmacokinetic parameters for dantrolene as well as its major metabolite 5-hydroxydantrolene following oral dose of 500 mg of either dantrolene capsules or paste to horses. Both formula-

tions appear to be comparable in terms of maximal and time to maximal plasma concentrations. Furthermore, we have demonstrated that the compound is rapidly metabolized to 5-hydroxy-dantrolene following oral administration to horses and analysis of urine samples show that parent and metabolite are below the limit of detection by 168 h postdantrolene administration. Based on the pharmacokinetics of dantrolene, a 48- and 168-h withdrawal guideline should be adopted for oral administration of Dantrium capsules for blood and urine, respectively. *In vitro* incubations with equine liver microsomes also produced 5-hydroxydantrolene metabolite seen *in vivo*. Furthermore, results from these studies suggest that hydroxylation of dantrolene in the horse is carried out by multiple cytochrome P450 enzymes, although further studies are necessary to identify the specific enzymes responsible.

ACKNOWLEDGMENTS

The authors thank Daniel McKemie and Jamie Deuel for technical assistance.

REFERENCES

- Court, M.H., Engelking, L.R., Dodman, N.H., Anwer, M.S., Seeler, D.C. & Clark, M. (1987) Pharmacokinetics of dantrolene sodium in horses. *Journal of Veterinary Pharmacology and Therapeutics*, **10**, 218–226.
- Edwards, J.G.T., Newton, J.R., Ramzan, P.H.L., Pilsworth, R.C. & Shepherd, M.C. (2003) The efficacy of dantrolene sodium in controlling exertional rhabdomyolysis in the thoroughbred racehorse. *Equine Veterinary Journal*, **35**, 707–710.
- Hollifield, R.D. & Conklin, J.D. (1973) Determination of dantrolene in biological specimens containing drug-related metabolites. *Journal of pharmaceutical sciences*, **62**, 271–274.
- Jayyosi, Z., Villoutreix, J., Ziegler, J.M., Batt, A.M., DeMaack, F., Siest, G. & Thomas, P.E. (1993) Identification of cytochrome P-450 isozymes involved in the hydroxylation of dantrolene by rat liver microsomes. *Drug Metabolism and Disposition*, **21**, 939–945.
- Krause, T., Gerbershagen, M.U., Fiege, M., WeiBhorn, R. & Wappler, F. (2004) Dantrolene-A review of its pharmacology, therapeutic use and new developments. *Anaesthesia*, **59**, 364–373.
- McKenzie, E.C., Valberg, S.J., Godden, S.M., Finno, C.J. & Murphy, M.J. (2004) Effect of oral administration of dantrolene sodium on serum creatinine kinase activity after exercise in horses with recurrent exertional rhabdomyolysis. *American Journal of Veterinary Research*, **65**, 74–79.
- Omura, T. & Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes. *Journal of Biological Chemistry*, **239**, 2370–2378.
- Wuis, E.W., Vree, T.B. & Van Der Kleijn, E. (1990) Pharmacokinetics of intravenously administered dantrolene and its 5-hydroxy metabolite in dogs. *International journal of clinical pharmacology research*, **10**, 203–210.

Dantrolene

Lynn Hovda

To: Heather Knych
Subject: RE: Dantrolene LOD and manuscript

-----Original Message-----

From: Heather Knych [mailto:hknych@ucdavis.edu]
Sent: Thursday, December 09, 2010 12:31 PM
To: Lynn.Hovda@state.mn.us
Subject: RE: Dantrolene LOD and manuscript

Hi Lynn

For our assay, the LOD for plasma was the same as the LOQ for both hydroxy dantrolene and dantrolene-that is 0.1 ng/mL.

For urine, the LODs are as you have below. We did not quantitate the urine dantrolene or hydroxy-dantrolene concentrations, meaning we did not do studies to determine what this would be-I just know that the concentrations were below the LOD of our analytical method by 168 hours post drug administration.

-----Original Message-----

From: Lynn Hovda [mailto:Lynn.Hovda@state.mn.us]
Sent: Thursday, December 09, 2010 7:13 AM
To: 'Heather Knych'
Subject: RE: Dantrolene LOD and manuscript

Heather:

Great to meet you as well. Please tell Birgit I said hi and when you meet Ahna Brutlag in January, you will love her. She is a joy.

I just need to make sure I understand this completely. And need some help with plasma.

Dose = 500 mg

Route = PO

LOD (Urine) = 20 ng/ml of hydroxyl metabolite LOD (Urine) = 50 ng/ml for dantrolene Withdrawal time = 168 hours

What of plasma??

LOD (plasma) = hydroxyl metabolite??

LOD (plasma) = dantrolene ???

Withdrawal time = 48 hours??

Many thanks and I hope you stayed warm yesterday.

Lynn Hovda

-----Original Message-----

Pharmacokinetics of detomidine and its metabolites following intravenous and intramuscular administration in horses

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Keywords: horse; pharmacokinetics; detomidine; sedation; alpha-2 agonist

Summary

Reasons for performing study: Detomidine is commonly used i.v. for sedation and analgesia in horses, but the pharmacokinetics and metabolism of this drug have not been well described.

Objectives: To describe the pharmacokinetics of detomidine and its metabolites, 3-hydroxy-detomidine (OH-detomidine) and detomidine 3-carboxylic acid (COOH-detomidine), after i.v. and i.m. administration of a single dose to horses.

Methods: Eight horses were used in a balanced crossover design study. In Phase 1, 4 horses received a single dose of i.v. detomidine, administered 30 µg/kg bwt and 4 a single dose i.m. 30 µg/kg bwt. In Phase 2, treatments were reversed. Plasma detomidine, OH-detomidine and COOH-detomidine were measured at predetermined time points using liquid chromatography-mass spectrometry.

Results: Following i.v. administration, detomidine was distributed rapidly and eliminated with a half-life ($t_{1/2(e)}$) of approximately 30 min. Following i.m. administration, detomidine was distributed and eliminated with $t_{1/2(e)}$ of approximately one hour. Following, i.v. administration, detomidine clearance had a mean, median and range of 12.41, 11.66 and 10.10–18.37 ml/min/kg bwt, respectively. Detomidine had a volume of distribution with the mean, median and range for i.v. administration of 470, 478 and 215–687 ml/kg bwt, respectively. OH-detomidine was detected sooner than COOH-detomidine; however, COOH-detomidine had a much greater area under the curve.

Conclusions and potential relevance: These pharmacokinetic parameters provide information necessary for determination of peak plasma concentrations and clearance of detomidine in mature horses. The results suggest that, when a longer duration of plasma concentration is warranted, the i.m. route should be considered.

Introduction

Detomidine (Dormosedan)¹ is a potent α_2 adrenoreceptor agonist commonly used for sedation and analgesia in horses (Wood *et al.* 1989). Due to its highly lipophilic behaviour, detomidine is rapidly absorbed, possesses a high affinity for the

central nervous system (Stanley *et al.* 1992) and is considered to be 50 times more potent than xylazine (Chui *et al.* 1992). Detomidine also has potent cardiopulmonary effects including increased incidence of AV block and bradycardia and increased pulmonary and systemic vascular resistance (Wagner *et al.* 1991). The analgesic (Chambers *et al.* 1993; England and Clarke 1996; Oijala and Katila 1998; Clark and Clark 1999) and cardiovascular effects (Sarazan *et al.* 1989; Wagner *et al.* 1991) of detomidine in the horse are well documented. However, studies describing the pharmacokinetics are limited (Salonen 1986; Salonen *et al.* 1989).

Initial pharmacological investigations were performed in a number of species including the horse (Salonen 1986). In one such study, a 50 µg/kg bwt i.v. dose was administered to a single animal and serum concentrations determined. A subsequent study described the disposition of detomidine (80 µg/kg bwt) following i.m. and i.v. administration to 6 horses (Salonen *et al.* 1989). In both studies detomidine identification was performed by radioimmunoassay (RIA). However, this method does not provide the sensitivity of the analytical methodology (liquid chromatography-mass spectrometry [LC-MS]), employed in the current study, or the ability to separate metabolites. In addition, clinically relevant doses were not administered and the pharmacokinetic parameters of detomidine metabolites were not determined (Salonen 1986; Salonen *et al.* 1989).

Detomidine has proved to be an extremely efficacious sedative and analgesic agent and, as such, is used routinely by equine clinicians. However, the potential for abuse of this drug in performance horses is high, leading the industry to regulate its use. Biological samples, including plasma and urine, are collected routinely and analysed by regulatory laboratories for the presence of the parent compound and its metabolites.

The purpose of the study presented here was to use highly sensitive LC-MS methodology to measure plasma concentrations and determine the pharmacokinetics of detomidine and its metabolites following a clinically applicable dose. It was hypothesised that LC-MS would increase sensitivity and decrease the limit of quantification (LOQ) compared to previously reported methodologies. Furthermore, it was predicted that metabolites would be detected for an extended period beyond the clearance of the parent compound following drug administration.

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Materials and methods

Horses

Eight healthy, mature horses, including 7 Thoroughbreds and one Thoroughbred/Quarter Horse cross (3 mares, 5 geldings, age mean \pm s.d. 5.3 ± 3.2 years and weight 531 ± 31 kg) were studied. The study was approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Experimental design

A balanced crossover design was used. In Phase 1, 4 horses received a single dose of detomidine, administered i.v. $30 \mu\text{g}/\text{kg}$ bwt and 4 horses received a single dose of detomidine, administered i.m. $30 \mu\text{g}/\text{kg}$ bwt. In Phase 2, the treatments were reversed. To ensure complete clearance of the drug, at least one week was allowed to elapse between Phases 1 and 2.

Instrumentation and drug administration

A 14 gauge catheter (Angiocath, 12 cm)² was placed in the left external jugular vein for sampling and a 18 gauge catheter (Angiocath, 5 cm)² in the right jugular vein for detomidine administration. The site of intramuscular (i.m.) administration was the neck muscles on the side contralateral to the sampling catheter. Each horse was weighed prior to drug administration. Baseline samples were collected and horses were administered $30 \mu\text{g}/\text{kg}$ bwt detomidine, either i.v. or i.m.

Blood (10 ml) was collected at baseline, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 10, 15, 30 and 45 min, and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 18, 24, 36 and 48 h post administration. Catheters were removed following collection of the 24 h sample and the remaining samples collected by direct venipuncture. Blood was centrifuged for 10 min and plasma stored at -20°C until analysis.

Measurements of plasma detomidine concentrations

Detomidine concentrations in test plasma were measured using LC-MS analysis of protein-precipitated samples. The calibration standards were prepared as follows: stock solutions were made by dissolving 10.0 mg detomidine standard in 10.0 ml of acetonitrile (ACN). Working solutions were prepared by dilution of the detomidine stock solution with ACN to concentrations of 100 and 500 ng/ml. Plasma calibrators were prepared by dilution of the working detomidine solutions with drug free plasma to concentrations of 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 50, 100 and 200 ng/ml. Calibration curves and negative control samples were freshly prepared for each quantitative assay. In addition, quality control samples (serum fortified with analytes at concentrations mid-point of the standard curve) were included routinely as an additional check of accuracy. The targeted LOQ was 0.2 ng/ml and the targeted limit of detection (LOD) was approximately 0.05 ng/ml. The concentration of detomidine in each sample was determined by the internal standard method using the peak area ratio and linear regression analysis.

Quantitative analyses were performed on a LTQ Linear Ion Trap Mass Spectrometer³ coupled with an 1100 Series Liquid Chromatography system⁴. Chromatography employed a Discovery HS C₁₈, 5 cm x 2.1 mm, 3 μm , column⁵ and a linear

gradient of ACN in water with a constant 0.2% formic acid at a flow rate of 0.4 ml/min. The ACN concentration was held at 10% for 0.4 min, ramped up to 90% over 7 min. Prior to analysis, plasma samples, standards and quality control sample were allowed to thaw at room temperature. The plasma proteins were extracted by precipitation with the addition of 0.5 ml ACN containing 10 ng/ml internal standard. All samples were vortex mixed for 1.5 min, followed by centrifugation (2000 *g* for 5 min, at 4°C). The injection volumes were 10.0 μl .

Detection and quantification employed electrospray ionization (ESI) full scan ms^2 transitions of initial precursor ion for detomidine (mass to charge ratio [*m/z*] 187). The response for the major product ions, for detomidine (*m/z* 81) was plotted and peaks at the proper retention time integrated using LCQuan software³, which was used to generate calibration curves and quantitate these analytes in all samples.

The concentration of detomidine in each sample (e.g. calibrators, quality control and unknowns) was determined by an internal standard method using the peak area ratio and linear regression analysis. The response for detomidine was linear and gave correlation coefficients (r^2) of 0.99 or better. The technique was optimised to provide a limit of detection at 0.05 ng/ml and limit of quantitation at 0.2 ng/ml. The percent recovery for detomidine at 1.0 ng/ml was 96% of the standard. Intraday accuracies (% of nominal concentration) were 94.1 and 96.6% for 1.0 and 50.0 ng/ml, respectively. Interday accuracies were 95.4 and 97.8% for 1.0 and 50.0 ng/ml, respectively. Intraday precisions (% relative standard deviation) were 2.6 and 1.8% for 1.0 and 50 ng/ml, respectively. Interday precisions were 4.7 and 3.5% for 1.0 and 50 ng/ml, respectively.

Detection and quantification employed ESI full scan ms^3 transitions of initial precursor ion for OH-detomidine (*m/z* 202) and ms^2 transitions of initial precursor ion for COOH-detomidine (*m/z* 217). The response for the major product ions, for OH-detomidine (*m/z* 185) and COOH-detomidine (*m/z* 199) were plotted and peaks at the proper retention time integrated using LCQuan software. The software was used to generate calibration curves and quantitate these analytes in all samples. The precision and accuracy parameters were <10% for both detomidine metabolites.

Pharmacokinetic analysis

Nonlinear least square regression was performed on plasma detomidine concentrations using WinNonlin Version 5.2 software⁶. Data were analysed using noncompartmental analysis (NCA) and compartmental modeling based on the Gauss-Newton (Levenberg and Hartley) method. Coefficient of variation of the pharmacokinetic parameters were generated and the area under the curve (AUC) of the NCA models were used as a reference to determine the best fit model and the appropriate weighting scheme for the individual horse data. Akaike information criterion (AIC) was used to determine the goodness of fit of models with different numbers of parameters for the same weighting scheme (Gabrielsson and Weiner 2000). Pharmacokinetic parameters for detomidine metabolites were generated using NCA based on a linear up/log down method. Bioavailability was determined by computing the ratio of i.m. AUC to i.v. AUC. Statistical analyses of pharmacokinetic parameters were performed with the Wilcoxon rank sum test using GraphPad Prism version 5.01 software⁷.

Results

Following i.v. and i.m. administration of detomidine, measurable amounts of the parent drug and both metabolites were detected. Detomidine was first detected at concentrations above the LOQ at 0.5–1.0 min and last detected at 3–4 h post administration of i.v. detomidine (Fig 1). Following i.v. administration, OH-detomidine and COOH-detomidine (Fig 2) were first detected at concentrations above the LOQ at 2.0–30 min and 15–30 min respectively, and last detected at 45–90 min and 8–12 h, respectively. After i.m. administration detomidine was first detected at concentrations above the LOQ at 1.5–45 min and last detected at 5–6 h (Fig 1). Following i.m. administration, OH-detomidine and COOH-detomidine (Fig 2) were first detected at concentrations above the LOQ at 30 min and 60–150 min, respectively, and last detected at 6 h and 8–12 h, respectively.

A one-compartment model best described the decrease in plasma detomidine concentrations over time following i.v. and i.m. administration (Fig 1). Detomidine concentrations declined rapidly with elimination half life ($t_{1/2(e)}$) of approximately 30 min following i.v. administration and one hour following i.m.

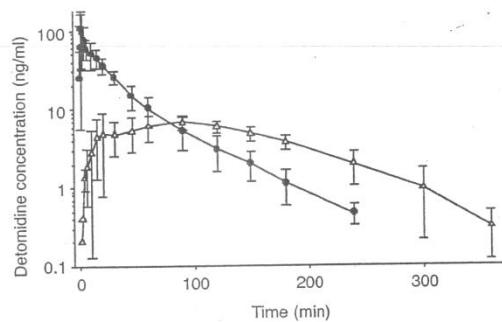


Fig 1: Semi-log scale: Mean \pm s.d. of plasma detomidine concentrations at predetermined time points after i.v. and i.m. administration of detomidine 30 μ g/kg bwt. Time of detomidine administration was designated as time 0. Data are incomplete beyond 360 min, and therefore are not included. \bullet = i.v.; Δ = i.m.

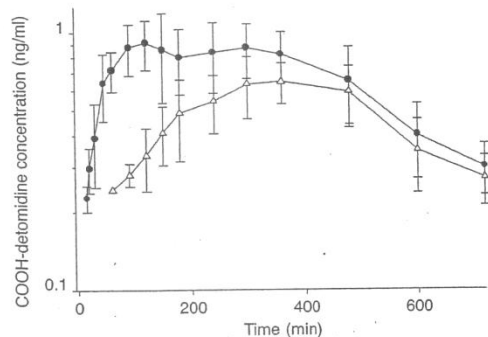


Fig 2: Semi-log scale: Mean \pm s.d. of plasma COOH-detomidine concentrations at various time points after i.v. and i.m. administration of detomidine. Time of detomidine administration was designated as time 0. \bullet = i.v.; Δ = i.m.

administration. The $AUC_{0 \rightarrow \infty}$ and maximum concentration (C_{max}) were significantly greater for i.v. vs. i.m. administration, while time of maximum concentration (T_{max}) was significantly greater following i.m. vs. i.v. administration (Table 1).

Following both administration routes, OH-detomidine was detected prior to COOH-detomidine. OH-detomidine plasma concentrations were below the LOQ for a number of the horses at numerous timepoints; therefore, pharmacokinetic parameters could be generated only for 5 of the 8 horses following i.v. administration and could not be generated from data obtained following i.m. administration. The mean, median and range for the $t_{1/2(e)}$ of OH-detomidine were 144.6, 66.32 and 34.45–326.8 min, respectively; and for the C_{max} of OH-detomidine were 0.42, 0.38 and 0.25–0.77 ng/ml, respectively. The mean, median and range for the T_{max} of OH-detomidine were 27, 30 and 6–45 min. The $t_{1/2(e)}$ of COOH-detomidine was approximately 200 min for both i.v. and i.m. administration. Similar to the parent compound, the $AUC_{0 \rightarrow \infty}$ and C_{max} of COOH-detomidine were significantly greater for i.v. vs. i.m. administration, while T_{max} was significantly greater following i.m. vs. i.v. administration (Table 2). The bioavailability of i.m. detomidine was calculated to be $54 \pm 14\%$.

Discussion

Analysis by LC-MS detected detomidine and both metabolites, OH-detomidine and COOH-detomidine, in plasma samples from

TABLE 1: Pharmacokinetic parameters for detomidine from 8 horses given 30 μ g/kg bwt detomidine i.v. or i.m.

	Intravenous			Intramuscular		
	Mean	Median	Range	Mean	Median	Range
AUC (min*ng/ml)	2495	2574	(1633–2970)	1311	1384	(959.3–1504)
$t_{1/2(e)}$ (min)	26.36	24.06	(12.68–41.26)	53.40	51.41	(35.85–90.09)
* C_{max} (ng/ml)	74.44	65.41	(43.70–139.3)	6.89	5.88	(4.04–10.7)
* T_{max} (min)	2.12	1.50	(1.0–6.0)	77.04	74.17	(51.72–130.0)
CL (ml/min/kg)	12.41	11.66	(10.10–18.37)			
Vd (ml/kg bwt)	470.5	478.6	(215.4–686.6)			
MRT (min)	38.71	39.65	(16.97–52.67)			

Values are expressed as mean, median (range). AUC = area under the plasma concentration-time curve; $t_{1/2(e)}$ = elimination half life; C_{max} = maximum observed plasma drug concentration; T_{max} = time after detomidine administration at which C_{max} was observed; CL = total body clearance; Vd = volume of distribution; MRT = mean residence time, extrapolated to the last time point. * C_{max} and T_{max} for i.v. PK parameters generated by noncompartmental analysis modelling since model used did not generate T_{max} parameter.

TABLE 2: Pharmacokinetic parameters for detomidine metabolites from 8 horses given 30 μ g/kg bwt detomidine i.v. or i.m.

	Intravenous COOH-detomidine			Intramuscular COOH-detomidine		
	Mean	Median	Range	Mean	Median	Range
AUC _{last} (min*ng/ml)	458.7	485.4	(215.7–581.5)	298.2	313.6	(189.3–415.1)
AUC _{0 \rightarrow \infty} (min*ng/ml)	542.3	551.6	(302.4–677.6)	410.5	411.7	(302.3–502.7)
$t_{1/2(e)}$ (min)	204.3	183.9	(168.0–284.8)	212.6	209.0	(152.6–257.3)
C_{max} (ng/ml)	1.05	1.07	(0.71–1.51)	0.69	0.69	(0.45–0.95)
T_{max} (min)	168.8	135.0	(90–300)	375	360	(300–480)

Values are expressed in means \pm s.d. $t_{1/2(e)}$ = elimination half life; T_{max} = time after detomidine administration at which C_{max} was observed; C_{max} = maximum observed plasma drug concentration; AUC_{0 \rightarrow \infty} = area under the plasma concentration-time curve, extrapolated to infinity; AUC_{last} = area under the plasma concentration-time curve, extrapolated to the last time point.

all 8 horses. Detomidine was rapidly absorbed and distributed and exhibited monophasic elimination.

In this study i.v. administration of detomidine led to a greater peak plasma concentration and AUC, while i.m. administration resulted in a greater $t_{1/2(e)}$ and time of maximum plasma concentration. Time of first detection of detomidine in plasma samples was variable following i.m. administration. One possible explanation for this is variability in absorption from the site of administration. OH-detomidine was detected prior to COOH-detomidine. This was to be expected as the biotransformation of detomidine involves an aliphatic hydroxylation of detomidine to generate OH-detomidine. The OH-detomidine undergoes a further dehydrogenation reaction to generate COOH-detomidine (Salonen *et al.* 1988).

The presence of measurable concentrations of COOH-detomidine well after those of the parent compound at a clinically applicable dose may be beneficial in the regulatory detection of detomidine in the performance horse. Further investigation is necessary to determine whether the metabolites themselves have any pharmacological effect.

The sensitivity of LC-MS has allowed for the detection and quantification of the parent drug and its metabolites at concentrations lower than previously investigated. The benefits of LC-MS include its sensitivity and selectivity. In addition, LC-MS provides more accurate results compared to RIA where higher amounts of parent compound can often be attributed to interferences between parent drug and metabolites (Etter *et al.* 2006). In one study comparing RIA to LC-MS, higher plasma concentrations of budesonide were reported with RIA, possibly due to cross reactivity of the metabolites with the antibody targeting parent compound (Dimova *et al.* 2003).

In contrast to previously published work that utilised RIA, compartmental analysis used in the present study indicates that both i.v. and i.m. administered detomidine is best described by a mono-exponential model demonstrating first order kinetics. Previous studies reported that detomidine metabolism is a biphasic process (Salonen 1986; Salonen *et al.* 1989). In the study by Salonen *et al.* (1989), however, some data could only be fitted to a mono-exponential model and, therefore, the mean concentration was used to determine pharmacokinetic parameters as opposed to using the data from individual animals. This may serve to explain the discrepancy between the Salonen and present studies. Salonen (1986) investigated the pharmacokinetics of detomidine in various species, including following administration of a 50 µg/kg bwt i.v. dose to a single horse. In contrast to our data, the total body clearance in Salonen's study was smaller and the $t_{1/2(e)}$ and volume of distribution larger. In a subsequent study by Salonen *et al.* (1989), detomidine (80 µg/kg bwt) was administered to 6 horses i.v. and 6 horses i.m. This study demonstrated detomidine, when administered i.v., was best described by a 2-compartment exponential equation. In addition, in the current study the $t_{1/2(e)}$ was approximately 2–3 times smaller than the value calculated by Salonen *et al.* (1989). Furthermore, the latter study reported a slower total body clearance and larger volume of distribution and AUC. For i.m. administration C_{max} and T_{max} were not generated using pharmacokinetic analysis, rather they were reported from observed data as 51.3 ng/ml and 0.50 h, respectively. The $t_{1/2(e)}$ and AUC were reported to be 1.78 h and 131.6 ng*h/ml (Salonen *et al.* 1989). These values are markedly different from the values generated in the present study with the AUC, C_{max} and $t_{1/2(e)}$

being much greater, and the T_{max} having markedly smaller values. These variations are probably multifactorial, including individual horse variation or differences in analytical technique and pharmacokinetic analysis.

The present study provides detailed pharmacokinetic information on the absorption, metabolism and elimination of detomidine in the horse, which allow regulatory veterinarians to establish definitive withdrawal guidelines for detomidine use in closely regulated industries. The combination of pharmacokinetic and pharmacodynamic data allows clinicians to develop administration regimens to target desired plasma concentrations and sedation effects. The pharmacodynamic properties of detomidine were also investigated concurrently during this study and these data are intended for submission in the near future.

Manufacturers' addresses

- ¹Pfizer, Granton, Connecticut, USA.
²Becton Dickinson Infusion Therapy System, Sandy, Utah, USA.
³Thermo Fisher Scientific, San Jose, California, USA.
⁴Agilent Technologies, Palo Alto, California, USA.
⁵Supelco, Bellefonte, Pennsylvania, USA.
⁶Pharsight Corporation, Mountain View, California, USA.
⁷GraphPad Software, La Jolla, California, USA.

Acknowledgements

The authors would like to thank Judy Sharp and Tiffany Snell for their assistance with the study and Heather Knych for her assistance with revisions of the paper. The authors would like to thank Pfizer Animal Health for their financial support in this study as well as their gift of detomidine metabolite reference standards. Additional funding was provided by the Buck Foundation, the Star Program and the Veterinary Scientist Training Program (K.N. Grimsrud).

References

- Chambers, J.P., Livingston, A., Waterman, A.E. and Goodship, A.E. (1993) Analgesic effects of detomidine in Thoroughbred horses with chronic tendon injury. *Res. vet. Sci.* **54**, 52–56.
- Chui, Y.C., Mutlib, A., Esaw, B., Zarate, R. and Young, L.M. (1992) Detection of detomidine and its metabolites in equine plasma and urine by LC/MS/MS. In: *Proceedings of the 9th International Conference of Racing Analysts and Veterinarians*. pp 85–92.
- Clark, J.O. and Clark T.P. (1999) Analgesia. *Vet. Clin. N. Am.: Equine Pract.* **15**, 705–723.
- Dimova, H., Wang, Y., Pommery, S., Moellmann, H. and Hochhaus, G. (2003) SPE/RIA vs. LC/MS for measurement of low levels of budesonide in plasma. *Biomed. Chromatogr.* **17**, 14–20.
- England, G.C.W. and Clarke, K.W. (1996) Alpha-2 adrenoceptor agonists in the horse: A review. *Br. vet. J.* **152**, 641–657.
- Etter, M.L., Eichhorst, J. and Lehotay, D.C. (2006) Clinical determination of 17-hydroprogesterone in serum by LC-MS/MS: Comparison to Coat-A-Count™ RIA method. *J. Chromatography B.* **840**, 69–74.
- Gabrielsson, J. and Weiner, D. (2000) Modeling strategies. In: *Pharmacokinetic and Pharmacodynamic Analysis: Concepts and Applications*, 4th edn., Swedish Pharmaceutical Press, Stockholm. p 460.
- Oijala, M. and Katila, T. (1998) Detomidine (Domosedan) in foals: sedative and analgesic effects. *Equine vet. J.* **20**, 327–330.
- Salonen, J.S. (1986) Pharmacokinetics of detomidine. *Acta vet. Scand.* **82**, 59–66.
- Salonen, J.S., Vaha-Vahe, T., Vainio, O. and Vakkuri, O. (1989) Single-dose pharmacokinetics of detomidine in the horse and cow. *J. vet. Pharmacol. Therap.* **12**, 65–72.
- Salonen, J.S., Vuorilehto, L., Eloranta, M. and Karjalainen, A. (1988) Metabolism of detomidine in the rat II. Characterization of metabolites in urine. *Eur. J. Drug Met. Pharmacokinetics* **13**, 59–65.

Sarazan, R.D., Starke, W.A., Krause, G.F. and Garner, H.E. (1989) Cardiovascular effects of detomidine, a new α_2 -adrenoceptor agonist, in the conscious pony. *J. vet. Pharmacol. Therap.* **12**, 378-388.

Stanley, S.D., Yang, J.-M., Wood, T.W., Salonen, J.S., Watt, D., Tai, H.-H. and Tobin, T. (1992) Detomidine clearance in the horse as determined by ELISA. In: *Proceedings of the 9th International Conference of Racing Analysts and Veterinarians*. pp 193-197.

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

Wood, T., Tai, C.L., Taylor, D.G., Woods, W.E., Wang, C.-J., Houtz, P.K., Tai, H.-H., Weckman, T.J., Yang, J.-M., Sturma, L., Chang, S.-L., Goodman, J.P., Stanley, S.D., Blake, J.W., Watt, D.S. and Tobin, T. (1989) Immunoassay detection of drugs in racing horses IX. Detection of detomidine in equine blood and urine by radioimmunoassay. *Res. Commun. Chem. Pathol. Pharmacol.* **63**, 263-279.

Author contributions The initiation, conception and planning of this study were by K.R.M. and S.D.S. Its execution was by K.N.G., K.R.M. and S.D.S., with statistics by K.N.G. All authors contributed to the writing of the paper.



The Swedish University of Agricultural Sciences (SLU)
Faculty of Veterinary Medicine and Animal Science announces a position
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SENIOR LECTURER, LARGE ANIMAL SURGERY
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The Faculty of Veterinary Medicine and Animal Science at SLU conducts research and education to promote the health and well-being of animals as well as humans and to develop a biologically and economically sustainable animal management. The faculty's research area comprises basic biomedical sciences, animal management, -health, and -welfare, veterinary medicine, veterinary public health and the influence of animals for the health and well-being of humans.

Subject description: Large animal surgery.

Duties: Overall responsibility for research and teaching in the subject area. The main task is teaching veterinary students theoretical and practical aspects of the discipline of large animal surgery, with emphasis on equine orthopaedic surgery. The clinical teaching will be performed in collaboration with clinical staff in Equine Clinic of the University Animal Hospital. The employment includes time for continuing education and for engagement in research projects within the department. Some administrative responsibilities at Department or Faculty level are probable. Moreover, dissemination of research results nationally and internationally and information within the subject area should be performed.

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Pharmacokinetics of detomidine and its metabolites following intravenous and intramuscular administration in horses

K. N. GRIMSRUD¹, K. R. MAMA², S. M. THOMASY¹, S. D. STANLEY^{1*}

Issue

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Abstract References Cited By

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Keywords: horse; pharmacokinetics; detomidine; sedation; alpha-2 agonist

Summary

Reasons for performing study: Detomidine is commonly used i.v. for sedation and analgesia in horses, but the pharmacokinetics and metabolism of this drug have not been well described.

Objectives: To describe the pharmacokinetics of detomidine and its metabolites, 3-hydroxy-detomidine (OH-detomidine) and detomidine 3-carboxylic acid (COOH-detomidine), after i.v. and i.m. administration of a single dose to horses.

Methods: Eight horses were used in a balanced crossover design study. In Phase 1, 4 horses received a single dose of i.v. detomidine, administered 30 µg/kg bwt and 4 a single dose i.m. 30 µg/kg bwt. In Phase 2, treatments were reversed. Plasma detomidine, OH-detomidine and COOH-detomidine were measured at predetermined time points using liquid chromatography-mass spectrometry.

Results: Following i.v. administration, detomidine was distributed rapidly and eliminated with a half-life ($t_{1/2\alpha}$) of approximately 30 min. Following i.m. administration, detomidine was distributed and eliminated with $t_{1/2\alpha}$ of approximately one hour. Following, i.v. administration, detomidine clearance had a mean, median and range of 12.41, 11.66 and 10.10–18.37 ml/min/kg bwt, respectively. Detomidine had a volume of distribution with the mean, median and range for i.v. administration of 470, 478 and 215–687 ml/kg bwt, respectively. OH-detomidine was detected sooner than COOH-detomidine; however, COOH-detomidine had a much greater area under the curve.

Conclusions and potential relevance: These pharmacokinetic parameters provide information necessary for determination of peak plasma concentrations and clearance of detomidine in mature horses. The results suggest that, when a longer duration of plasma concentration is warranted, the i.m. route should be considered.

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Detomidine/Rylam

Lynn Hovda

To: scot waterman
Subject: RE: cimetidine, omeprazole and ranitidine

Exhibit 27

From: scot waterman [mailto:swaterman@rmtcnet.com]
Sent: Monday, December 13, 2010 2:53 PM
To: Lynn.Hovda@state.mn.us
Subject: RE: cimetidine, omeprazole and ranitidine

- Detomidine: 10 ng/ml in urine of the metabolite (believe it is the hydroxy metabolite)...0.2 mg/kg IV; 48 hours
- Lidocaine: 25 ng/ml of the metabolite in urine...50 mg SQ; 96 hours
- Pyrilamine: 50 ng/ml in urine....up to 7 grams orally; 96 hours

From: Lynn Hovda [mailto:Lynn.Hovda@state.mn.us]
Sent: Thursday, December 09, 2010 4:07 PM
To: swaterman@rmtcnet.com
Subject: cimetidine, omeprazole and ranitidine

Scot:

Apparently our 48 hour rule is in statute and only the NSAIDS can be 24 hours. So...I need a regulatory limit for cimetidine, omeprazole, and ranitidine, not just the dose and dosing interval, as they now must be 48 hours. I know, I know, I know, but if you have something please let me know.

Otherwise, I did just get Heather and Scott's dantrolene info and Rick helped me out with the oral methocarbamol info.

Lynn

Lynn R. Hovda, DVM, MS, ACVIM
Chief Commission Veterinarian
Minnesota Racing Commission
PO Box 630
Shakopee, MN 55379
952-496-6487 (season); 952-496-7950 (off season)

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**Summary of Medication Recommendations
Pennsylvania Horse Racing Commission**

Updated September 8, 2010

- Anabolic & Androgenic Steroids
- Glucocorticoids (triamcinolone acetoneide, dexamethasone, methylprednisolone acetate)
- NSAID (flunixin, naproxen, nabumetone)
- Methocarbamol (IV)
- Dimethyl Sulfoxide (DMSO)
- Multiple NSAID (Stacking)
- NSAID (phenylbutazone updated)

1. Anabolic and Androgenic steroids.

As of October 1st, 2008, the Pennsylvania Horse Racing Commission prohibited the use anabolic and androgenic steroid in all horses competing in the State of Pennsylvania. No anabolic steroids (ABS) will be allowed in horses competing in the State of Pennsylvania. The exceptions are 100 pg/ml of plasma for boldenone, nandrolone, and stanozolol. Testosterone plasma concentrations greater than 100 pg/mL plasma is not allowed in the female or gelded male horse. Acceptable plasma concentrations of natural occurring testosterone of 2000 pg/ml and 500 pg/ml nandrolone have been established for the intact male horse.

Elimination from plasma of anabolic and androgenic steroids following a single intramuscular injection can be variable and a period of at least 30 to 60 days should be allowed before entering a horse in an official race (**Figures 1 and 2**). To assure compliance out-of-competition testing for anabolic and androgenic steroids is available in the State of Pennsylvania. Information for submitting an unofficial out-of-competition plasma sample for anabolic or androgenic steroids is available at all Pennsylvania Tracks.

Human Chorionic Gonadatrophin (HCG) provocative test is available to determine if a gelding is truly a gelding and not a monorchid, cryptorchid or has some remaining residual testicular tissue. Contact a Commission Veterinarian or Commission track manager for more information.

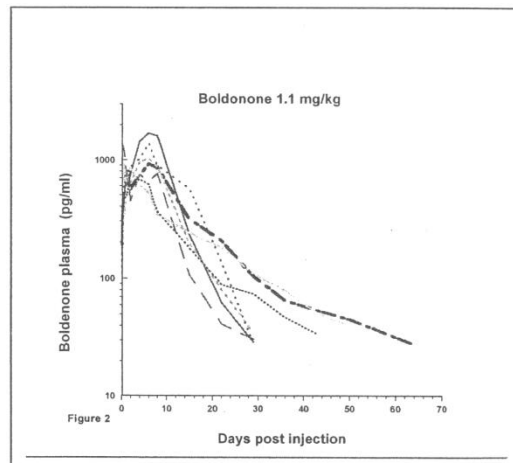
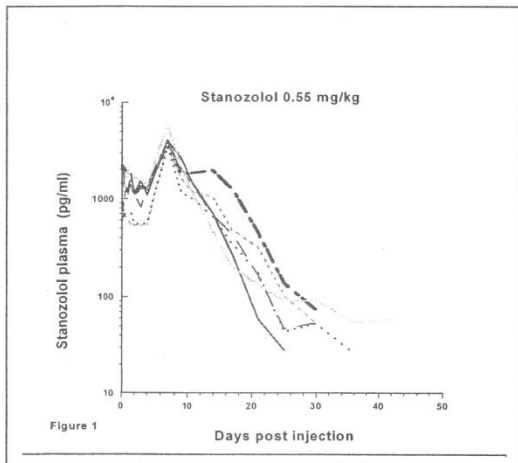


Figure 1 Plasma concentrations of stanozolol following the IM injection of 0.55 mg/kg in 7 horses.
Figure 2 Plasma concentrations of boldenone following the IM injection of 1.1 mg/kg in 6 horses.

References:

-
- Guan F, Uboh CE, Soma LR, Luo Y, Rudy J and Tobin T (2005) Detection, quantification and confirmation of anabolic steroids in equine plasma by liquid chromatography integrated with tandem mass spectrometry *Journal of Chromatography B* **829**:56-68.
- Soma LR, Uboh CE, Guan F and McDonnell S (2008) Plasma concentrations of testosterone and 19-nortestosterone (nandrolone) in the non-racing intact male horse by liquid chromatography-mass spectrometry. *Journal of Veterinary Pharmacology & Therapeutics* **31**:587-590.
- Soma LR, Uboh CE, Guan F, McDonnell S and Pack J (2007) Pharmacokinetics of Boldenone and Stanozolol and the Results of Quantification of Anabolic and Androgenic Steroids and in Race Horses and Non-Race Horses. *J. Vet. Pharmacol. Therap.* **30**:1-8.

2. Glucocorticoids: As of June 1st, 2009, the Pennsylvania Horse Racing Commission is regulating the intra-articular injections of glucocorticoids, to no less than 7 days prior to race-day. This policy dictates that the plasma concentration of all the exogenously administered glucocorticoids must be below the level of quantification.

Plasma concentration of triamcinolone acetonide (Vetalog[®]) (20 mg) and methylprednisolone acetate (Depo-Medrol[®]) (200 mg) administered via the intraarticular route were below the level of quantification at 5 and 7 days, respectively.

Plasma concentration of the intramuscular administration of triamcinolone acetonide (Vetalog[®]) at a dose of 20 mg was quantified in plasma for up to 14 days. It is recommended that this route of administration for Vetalog[®] and Depo-Medrol[®] be discontinued.

Plasma concentration of the intravenous administration of dexamethasone (Aziium solution) (25 mg) was below the level of quantification at 48 hours post injection.

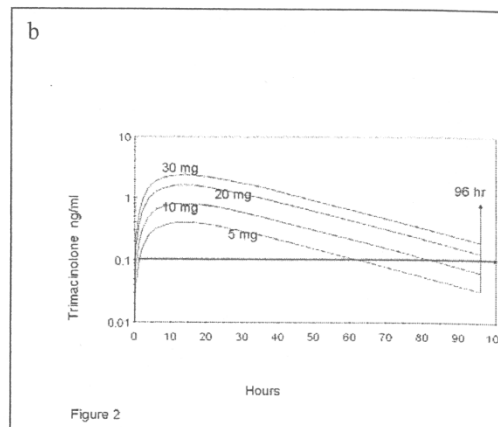
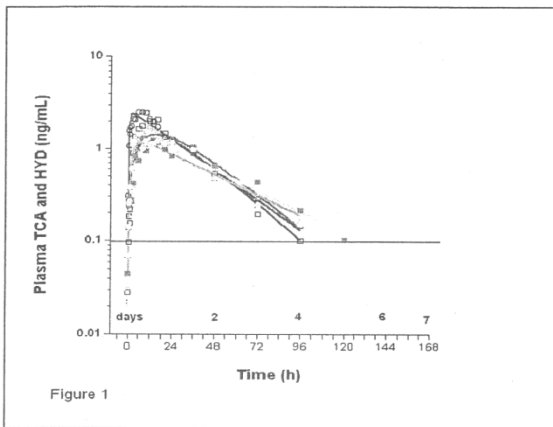
Plasma concentration of the intravenous administration of triamcinolone (20 mg) was below the level of quantification at 48 hours post injection.

See illustrations below

Triamcinolone acetonide (Vetalog[®]) - IA administrations.

Figure 1. Data points of the administration of 20 mg IA of triamcinolone acetonide to 6 horses. The solid line is the level of quantification (0.1 ng/ml).

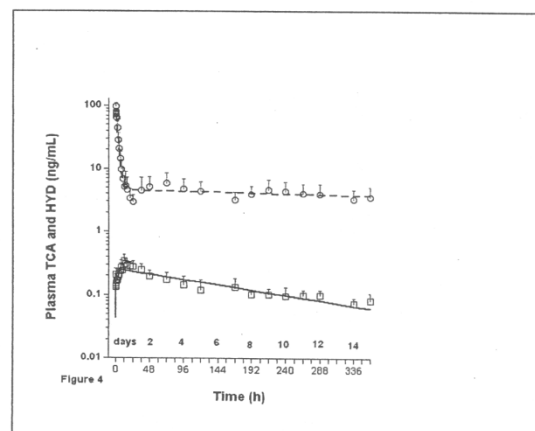
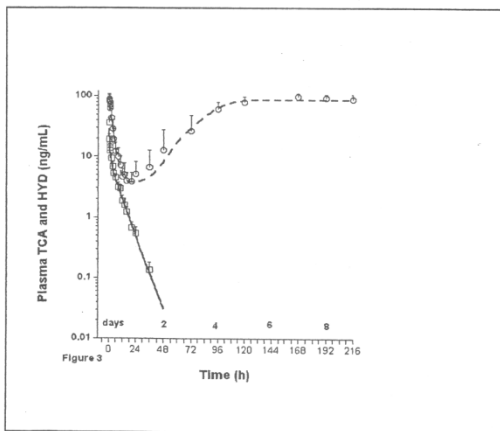
Figure 2. Simulation of plasma concentration of 5, 10, and 30 mg of triamcinolone acetonide base on the 20 mg dose administered IA. The solid line is the level of quantification (0.1 ng/ml).



Triamcinolone acetonide (Vetalog®) - IV and IM

Figure 3. Plasma concentration of triamcinolone acetonide (TA) (—□—) following the IV injection of 0.04 mg/kg and changes in endogenous hydrocortisone (—○—) from baseline (0 hours). Suppression and recovery of plasma hydrocortisone concentration are illustrated relative to the changes in the plasma concentration of TA (Mean and SD).

Figure 4. Plasma concentration of triamcinolone acetonide (TA) (—□—) following the IM injection of 0.04 mg/kg and changes in endogenous hydrocortisone (—○—) from baseline (time 0 hours). Suppression and no recovery of plasma hydrocortisone concentration are illustrated relative to the changes in the plasma concentration of TA (Mean and SD).



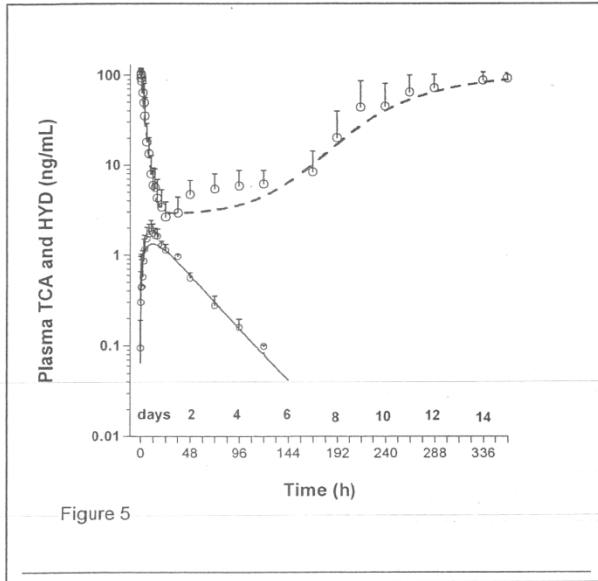
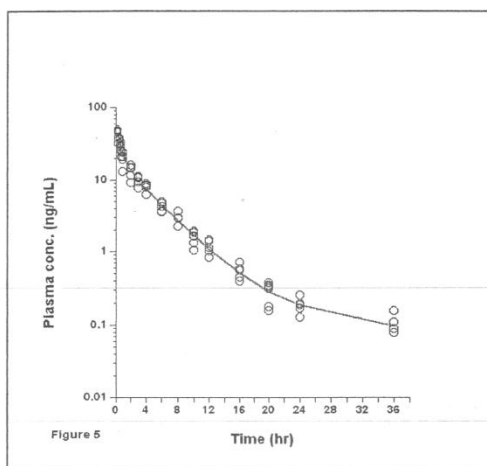


Figure 5. Plasma concentration of triamcinolone acetonide (TA) (—□—) following the IA injection of 0.04 mg/kg and changes of endogenous HYD (—○—) from baseline (0 hours). Suppression and recovery of plasma HYD concentration are illustrated relative to the changes in the plasma concentration of TA. Solid and dashed lines are the best fits for plasma TA and HYD concentrations, respectively. Mean and SD of 6 horses.

Dexamethasone (Azium®) - IV

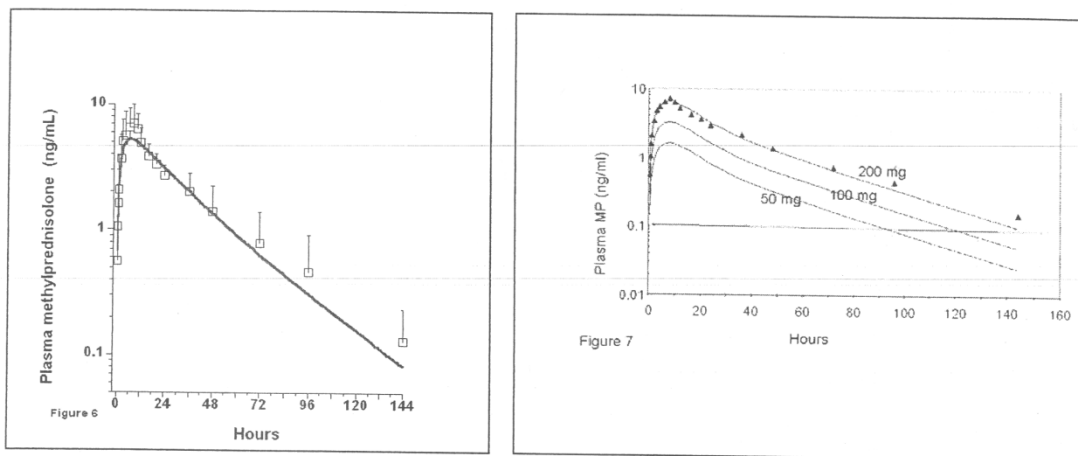
Figure 5a. Plasma concentration of DXM (—) following IV administration of 25 mg (0.05mg/kg). Solid line is the population mean best fit for DXM.



Methylprednisolone acetate (Depo-Medrol®) administered IA

Figure 6. Plasma concentration of MP (—) following administration of 200 mg IA. Solid line is the population mean best fit for MP (Mean and SD).

Figure 7. Simulation of plasma concentration of 100 and 50 mg of methylprednisolone acetate base on the 200 mg dose administered IA. The solid line is the level of quantification (0.1ng/ml).



References:

- Luo Y, Uboh CE, Soma LR, Guan FY, Rudy JA and Tsang DS (2005b) Simultaneous analysis of twenty-one glucocorticoids in equine plasma by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **19**:1245-1256.
- Soma LR, Uboh CE, Luo Y, Guan F, Moate PJ and Boston RC (2005) Pharmacokinetics of dexamethasone with pharmacokinetic/pharmacodynamic model of the effect of dexamethasone on endogenous hydrocortisone and cortisone in the horse. *Journal of Veterinary Pharmacology & Therapeutics* **28**:71-80.
- Soma LR, Uboh CE, Luo Y, Guan F, Moate PJ, Boston RC, Soma LR, Uboh CE, Luo Y, Guan F, Moate PJ and Boston RC (2006) Pharmacokinetics of methylprednisolone acetate after intra-articular administration and its effect on endogenous hydrocortisone and cortisone secretion in horses. *American Journal of Veterinary Research* **67**:654-662.

Flunixin:

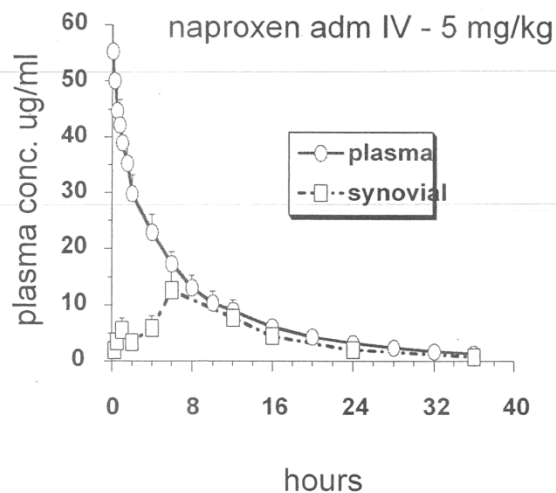
Pennsylvania Horse Racing Commission dose not consider the finding of flunixin in urine a violation. The corresponding plasma sample is analyzed by LC-MS and is a violation when the plasma concentration is in excess of 20 ng/ml. Flunixin at 1.1 mg/kg administered IV 24 hours prior to race day should not result in a violation.

The IM or SQ administration may result in a violation, due to a slower absorption pattern in the horse. Oral paste formulations should not be used. If oral route of administration is selected and the preparation is fed

in grain, the grain should be removed immediately following consumption to avoid later consumption of small amounts of medication remaining in the feed bucket.

Naproxen

Naproxen is a nonsteroidal anti-inflammatory drug (NSAID) used for the treatment of myositis and other inflammatory conditions in horses. In horses with experimentally induced myositis, naproxen was considered to be therapeutically more effective than phenylbutazone in providing relief of inflammatory swelling and the associated lameness. The recommended dose is 5 mg/kg IV and 10 mg/kg orally twice daily for 14 days. The reported bioavailability in the horse is 50 %.



Plasma and synovial concentrations of naproxen after IV administration of 5.0 mg/kg (~ 2.5 grams/horse) is shown in **Figure 1**. The highest plasma concentration of naproxen was 55.3 ug/ml at 5 minutes and at 48 hours after its administration the plasma concentration was 0.61 ug/ml.

figure 1

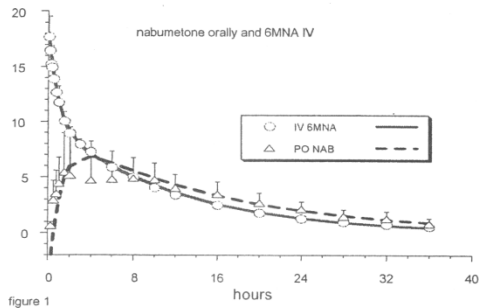
Recommendation: At least 48 hours should be allowed following a single IV dose of 5 mg/kg.

References

Soma LR, Uboh CE, Rudy JA, Perkowski SZ. Plasma and synovial fluid kinetics, disposition and urinary excretion of naproxen in the horse. *Am J Vet Res* 56(8):1075-1080, 1995.

Nabumetone

This drug has a similar structure to naproxen and due to the long half life of nabumetone at least 48 hours should be allowed following a single oral dose of 3.7 mg/kg.



Following the oral administration of nabumetone, (Figure 1) the drug is absorbed from the gastrointestinal tract and is metabolized to 6MNA. The plasma concentration of 6MNA after oral administration of 3.7 mg/kg of nabumetone peaked at ~2 to 3 hours, with concentrations of ~5.4 ug/ml. The absorption half-life ranged from 0.42 to 2.48 hours. Elimination half-life of the metabolite 6MNA was 11 hours with a range of 9.3 to 12.7 hours. The elimination half-life was slightly longer than that of naproxen.

References

Soma LR, Uboh C, Rudy J, Smith M. Disposition and excretion of 6MNA in the Horse: The Active Metabolite of Nabumetone. *Am J Vet Res* 57(4) 517-521, 1995.

Methocarbamol

Pennsylvania Horse Racing Commissions do not allow the administration of any drug within 24 hours of the post-time of the first race. The figure below shows the plasma concentration vs time curves of the administration of 2.2 mg/kg (100 mg/100 lbs of body wt) to 6 horses. The formulation of methocarbamol was from a compounding pharmacy with a labeled concentration of 100 mg/ml. The solution in which the drug was dissolved was not indicated on the label.

The range of total doses administered in the study was 1111 to 1265 mg by IV. Methocarbamol is rapidly eliminated with a mean terminal elimination half-life of 1.6 h (range 1.4 to 2.0 h). The Limit of Quantification (LOQ) is 1 ng/ml and the plasma concentration at 24 hours must be below the LOQ. In the administration of methocarbamol, a number of factors must be taken into consideration; the administration must be performed outside the 24-hour period, the variable weights of the horses being medicated and the knowledge that the solution being administered is not from an FDA approved company with consistent quality control standards.

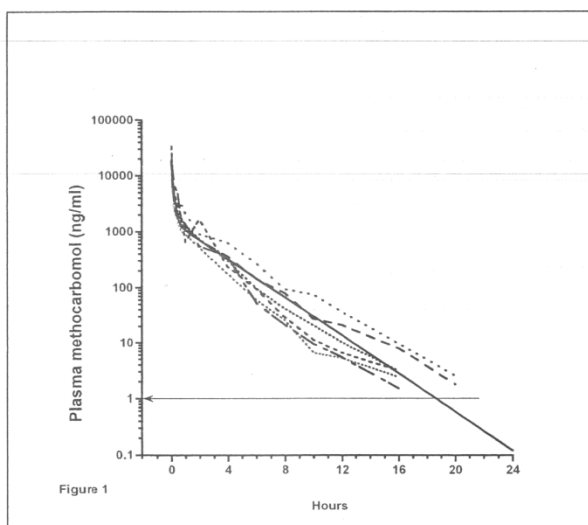


Figure 1. The plasma concentration vs time curves of the administration of 2.2 mg/kg (100 mg/100 lbs of body wt) to 6 horses. The solid line is the line of best fit of the 6 horses. The limit of quantification of the method is 1 ng/ml.

Dimethyl sulfoxide (DMSO).

Dimethyl sulfoxide (DMSO) is an ARCI Class 5 drug. This class includes those therapeutic medications for which concentration limits have been established by member racing jurisdictions as determined by their respective regulatory bodies. The Pennsylvania Harness Racing Commission has established guidelines for the topical administration of DMSO. Dimethyl sulfoxide is approved by the FDA for topical use.

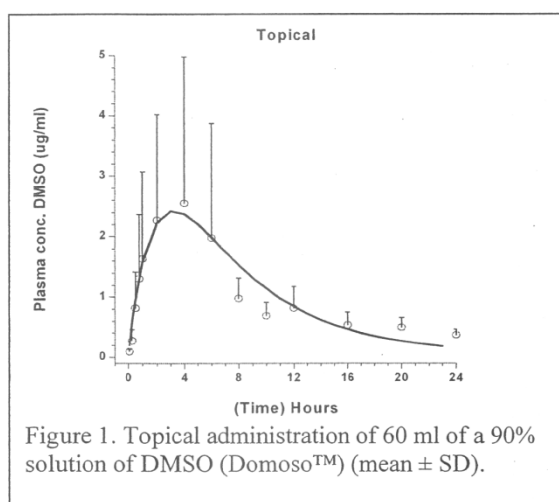
As of September 15, 2010, the **Pennsylvania Horse Racing Commission** will allow the topical administration of DMSO on race day. Oral or intravenous administration is prohibited on race day.

Dimethyl sulfoxide (DMSO) is a highly polar, water-miscible, hygroscopic substance that has been used for a number of medical purposes based on a broad range of incompletely understood pharmacological properties. It has been used as an anti-inflammatory agent and topically as a vehicle for systemic and local therapy with other drugs. Part of its therapeutic use is based on its ability to scavenge oxygen-free radicals which are implicated in tissue damage after the tissue insult. Based on these actions it has been administered IV for the treatment of central nervous system diseases, head trauma, brain and spinal cord trauma/edema. Dimethyl sulfoxide (DMSO) administrations.

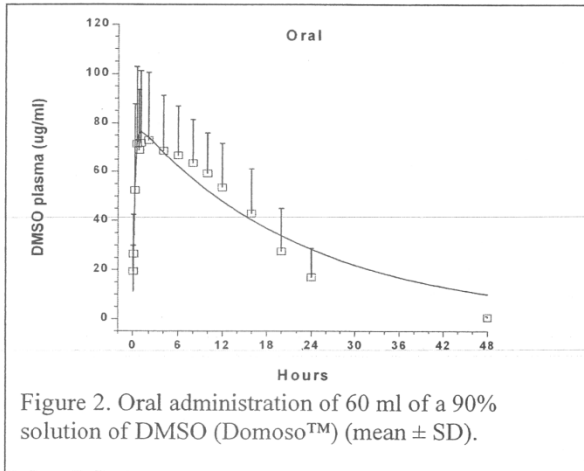
Nine adult male and female horse 4 to 10 years old and weighing 504.2 ± 19.0 kg, were used in a cross-over study design; where all horses received intravenous (IV), oral (PO) and topical (TOP) DMSO in random order. Sixty ml of medical grade 90% dimethyl sulfoxide (Domoso™) solution was used. To prevent hemolysis of red blood cells during the IV infusion, 60 ml of DMSO was diluted in 1000 ml of a sodium chloride solution and administered over 15 minutes. Sixty ml of DMSO was mixed with molasses in a dose syringe and administered PO. For the topical administration 60 ml of DMSO was rubbed on the rump and back area, covered with plastic sheet and a blanket to maximize the contact with the skin. The blanket was left on for 24 hours. A horizontal surface was selected for the topical administration to maximize the surface area and to insure the skin was exposed to 60 mL of DMSO.

Results.

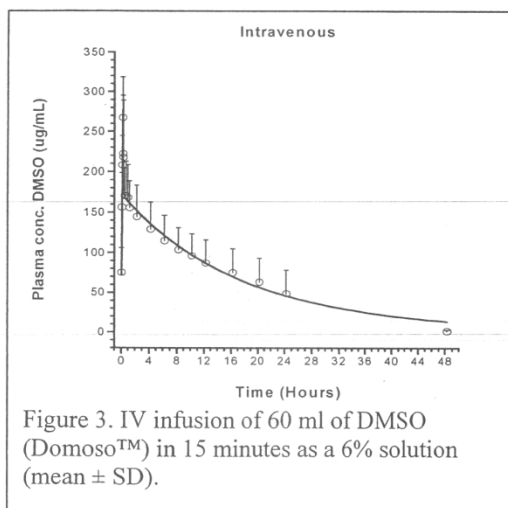
Topical administration. Following TOP administration the absorption half-life was 1.2 hours with a terminal elimination half-life of 4.5 hours. The peak plasma concentrations of DMSO occurred at 2 to 4 hours and were 1.2 to $8.2 \mu\text{g/ml}$. Compared to the IV administration the fractional absorption (bioavailability) was only 1.3 % of the dose administered (Figure 1). At 24 and 48 hours, 6 of the 9 horses had quantifiable concentrations of DMSO at concentrations of 0.36 (0.26-0.48) and 0.11 (0.03-0.19) $\mu\text{g/ml}$, respectively.



Oral administration. Following PO administration the absorption was rapid at a half-life of 0.15 hours with a terminal elimination of 15.5 hours. The peak plasma concentrations of DMSO occurred at 0.25 to 4 hours and were 47.4 to 129.9 $\mu\text{g}/\text{mL}$. Compared to the IV administration the fractional absorption (bioavailability) was 50.6 % of the dose administered (Figure 2). At 48 hours DMSO was quantified in 6 of the 9 horses at a mean plasma concentration of 0.66 (0.31-1.7 $\mu\text{g}/\text{ml}$).



Intravenous administration. Following 15 minute intravenous infusion of 60 ml of DMSO as a 10 % solution there was a rapid initial decline in the plasma concentration. This was a combination of tissue distribution and elimination through the pulmonary system. Terminal elimination half-life was 14.1 hours. Peak plasma concentrations of DMSO at 2 minutes after the infusion were 196.7 to 739.2 μ g/mL. At 48 hours DMSO was quantified in 7 of the 9 horses at a mean plasma concentration of 1.5 (0.1-3.4 μ g/ml).



Recommendations: Topical administration of DMSO will be allowed on race day with a threshold concentration of 10 μ g/ml. Following the oral or intravenous administration horses should not be entered into a race for 48 hours post administration. These guidelines are based on the administration of 60 ml topically, orally and intravenously.

Multiple NSAID (Stacking)

Stacking: Pennsylvania Horse Racing Commission does not allow the presence of 2 non-steroidal anti-inflammatory drugs (NSAID) or “stacking” on race day. If both are used for treatment during training and phenylbutazone is the NSAID of choice on race day flunixin should be withdrawn 48 hours prior to racing. If flunixin is the NSAID of choice on race day phenylbutazone should be withdrawn 72 hours prior to racing.

Phenylbutazone (PBZ)

The Pennsylvania Horse Racing Commission has announced that as of September 15, 2010 the permissible plasma concentration of phenylbutazone (PBZ) and its major metabolite oxyphenbutazone (OPBZ) is 2 micrograms (μ g/ml).

The majority of studies suggest an effect of PBZ at 24 hours at 4.4 mg/kg (2000 mg). This reflects and substantiates the opinion of many clinical veterinarians, many of whom will not perform a pre-purchase lameness examination unless the horse was free of non-steroidal anti-inflammatory drugs. This remains the opinion of many Commission Veterinarians in that they wish to examine a horse pre-race without the possibility

of an NSAID interfering with the examination and masking possible underlying musculoskeletal conditions. Based on scientific studies, residual effects of PBZ remain at 24 hours following administration of the above dose. The impact of sustained effect on the health, safety and welfare of the horse and its contribution to injuries during competition remains problematic.

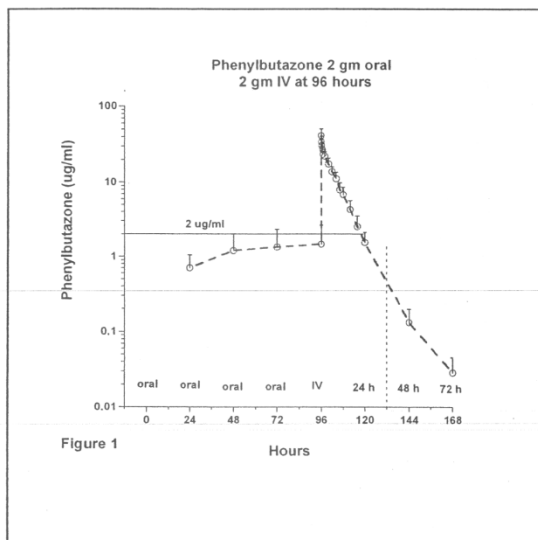


Figure 1 shows the results of daily oral administration of 2 grams of PBZ (4.4mg/kg) for 4 daily doses followed by 4.4mg/kg (2 grams) IV administration at 24 hours prior to post time. The 24, 48, and 72 hours post-administration are also shown in the figure. The plasma concentrations at 24 hours in these 6 horses were 1.6 (range of 0.9 to 2.6) $\mu\text{g/ml}$ and at 48 hours 0.11 (range of 0.06 to 0.23) $\mu\text{g/ml}$. The 24 hour plasma concentration in this group of horses was similar to the plasma concentrations found in the majority of post-race samples from PA Thoroughbred racetracks. The figure also indicated that following daily oral administrations a near steady state was achieved between 72 and 96 hours.

To continue this dosage schedule of PBZ administration, the IV dose should be administered at least 36 hours before post-time (see dotted line). It must be appreciated that the oral administration of PBZ 36 hours prior to post-time may not produce the same results as the IV administration based on the variability of the absorption of the drug from the stomach and gastrointestinal track. Thus, the practice involving oral administrations 24 prior to race-time is not recommended.

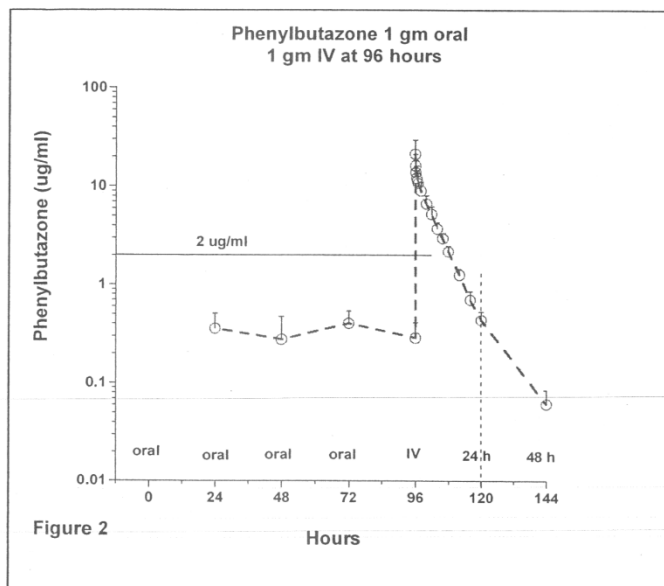


Figure 2 shows the results of daily oral administration of 1 grams of phenylbutazone (PBZ) (2.2mg/kg) for 4 daily doses followed by 2.2mg/kg (1 gram) IV administration at 24 hours prior to post time. The 48 hour post-administration plasma concentration are also shown in the figure. The plasma concentrations at 24 hours in 6 horses were 0.43 (range of 0.32 to 0.54) $\mu\text{g/ml}$ and at 48 hours it was 0.06 (range of 0.04 to 0.10) $\mu\text{g/ml}$.

At the dose of 1 gram by IV 24 hours prior to post time, the plasma concentrations of PBZ at 24 hours were all below 2 $\mu\text{g/ml}$.

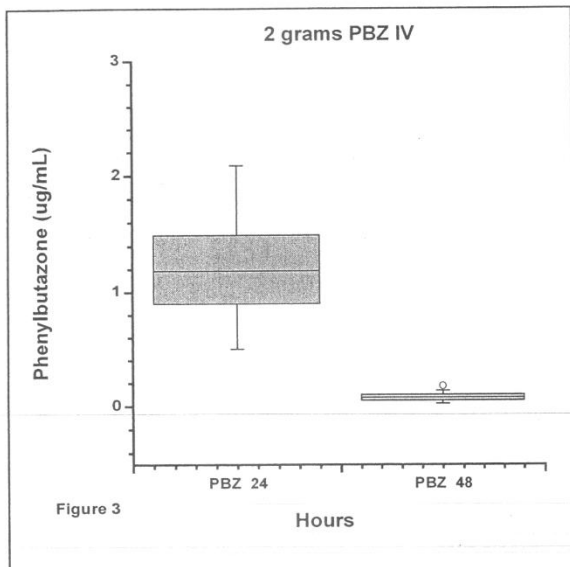


Figure 3 shows the results of a single IV administration of 2 grams (4.4mg/kg) of phenylbutazone (PBZ). This group of 20 horses did not have prior administration of PBZ. The 24 hour mean post-administration plasma concentration was 1.2 (range of 0.5 to 2.1) $\mu\text{g/ml}$ and mean 48 hours plasma concentration was 0.08 (range of 0.02 to 0.18) $\mu\text{g/ml}$.

These data indicate that even with no prior administrations of PBZ a dose of 2 grams 24 hours prior to post-time may produce a violation of the 2 $\mu\text{g/ml}$ threshold. It is suggested that if a dose of 4.4 mg/kg is to be administered that the weight of the horse be taken into consideration or PBZ be administered at 36 hours prior to race time.

Phenylbutazone IV administration to 20 horses (courtesy of the Drs Sams and Callahan of the University of Florida).

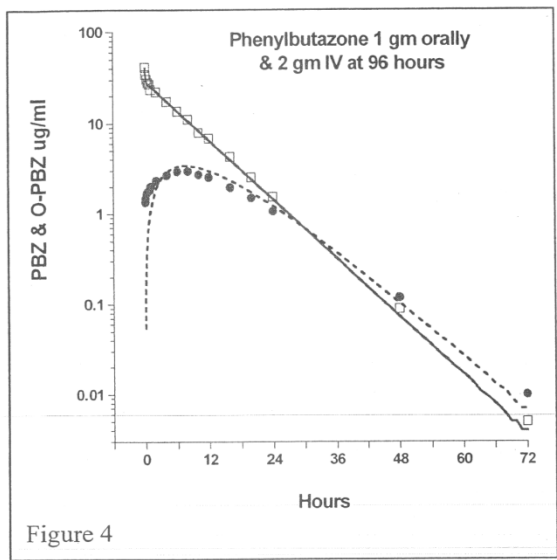


Figure 4

Figure 4 shows the relationship between PBZ (open squares) and the major metabolite OBBZ (filled circles). At 24, 48, and 72 hours the plasma concentrations were not significantly different. Oxyphenbutazone has anti-inflammatory properties.

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Urinary and Serum Concentrations of Diclofenac after Topical Application to Horses*

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CLINICAL RELEVANCE

The liposomal cream formulation of the NSAID diclofenac, which is approved by the FDA for use in horses, has been shown to be an effective, safe, and convenient way to treat localized areas of inflammation in horses. The results of this study revealed urinary and serum concentrations of diclofenac after topical administration of 1% liposomal diclofenac cream for 10 days at the labeled dose and at 2× and 4× the labeled dose. These results demonstrate the slow absorption and elimination of 1% liposomal diclofenac cream and may be useful when estimating the withdrawal time needed before a competition to prevent an inadvertent positive drug test.

INTRODUCTION

Veterinarians commonly administer NSAIDs to horses for the treatment of a wide variety of inflammatory conditions. Systemic administration was the standard of care in the past,

even if the inflammation was limited to a small anatomic area. The side effects of systemic NSAID administration, however, include gastrointestinal ulceration, renal toxicity, and injection-site reactions.¹ In contrast, topical NSAID administration is associated with fewer side effects, primarily because the total dose administered is far below the toxic threshold while therapeutic drug concentrations are still

*Funding for this study was provided by IDEXX Pharmaceuticals, Inc., Greensboro, NC.

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achieved locally. Topical NSAID administration in humans is efficacious for the treatment of rheumatoid arthritis, osteoarthritis, and other inflammatory conditions and has an improved safety profile compared with orally administered agents.^{2,3}

The phenylacetic acid NSAID diclofenac sodium is a nonselective cyclooxygenase and lipoxygenase inhibitor that is commonly used in human medicine (Voltaren, Novartis Phar-

particularly relevant to veterinarians treating racehorses or performance horses that are routinely subjected to drug-testing programs. Although the FDA-approved label indicates that diclofenac cream should be applied to only one joint twice daily, it is possible that equine practitioners will occasionally treat multiple sites on a single horse. Therefore, the objectives of this study were to determine the concentrations of diclofenac in serum and urine samples collect-

Topical NSAID administration is associated with fewer side effects than systemic administration.

maceuticals).⁴ The safety profile of topical diclofenac has been clearly established in humans; its efficacy is equivalent or superior to that of other commonly used NSAIDs, while the risk for gastrointestinal irritation and renal toxicity is relatively low.^{5,6}

Although there is not a diclofenac product approved for oral or parenteral administration in horses, the FDA recently approved a 1% diclofenac liposomal cream formulation (Surpass [1% diclofenac sodium] Topical Anti-Inflammatory Cream, IDEXX Pharmaceuticals) for topical administration to horses for control of joint pain and inflammation associated with osteoarthritis. The results of a clinical field trial indicate that the product is safe, easy to use, and effective in reducing lameness caused by degenerative joint disease.⁷ Therefore, this NSAID cream provides a new therapeutic modality for veterinarians to use in the treatment of a variety of musculoskeletal injuries and inflammatory conditions.

Little is known regarding systemic absorption and elimination of this 1% diclofenac liposomal cream when applied according to the label recommendations or at extra-label dosages. The elimination pattern of this agent is

ed from horses after topical administration of 1% liposomal diclofenac cream at 1×, 2×, and 4× the recommended label dose.

MATERIALS AND METHODS

Subjects and Treatments

Six Thoroughbred geldings, 5 to 11 years of age and weighing 520 to 587 kg, were used in this study, which was approved by the University of Florida Institutional Animal Care and Use Committee. The horses were healthy on physical examination at the start of the study. They were exercised on a high-speed treadmill three times a week and maintained on pasture with free access to water and mixed hay. The same six horses were used for the three consecutive treatments, which consisted of the application of 7.2 g of 1% diclofenac cream to one, two, or all four fetlocks twice daily for 10 days. Because of the prolonged treatment period, it was assumed that systemic residual drug concentrations after one treatment would have no significant effect on the ultimate urine and serum concentrations produced by the following treatment. Therefore, a minimum withdrawal period of 3 days between the final application of one treatment

protocol and the first application of the next was determined to be adequate for the purposes of this study.

For the first treatment protocol, one front fetlock of each horse was treated with 7.2 g of the product (i.e., 1× the label dose) twice daily for 10 days, and urine and blood samples were collected before treatment and 0.25, 8, 12, 24, 48, and 72 hours after the last treatment was applied. For the second treatment protocol, two fetlocks of each horse were treated with 7.2 g of product (i.e., 2× the label dose) twice daily for 10 days, and urine and blood samples were collected as previously described. For the third protocol, all four fetlocks of each horse were treated with 7.2 g of product (i.e., 4× the label dose) twice daily for 10 days, and urine and blood samples were

Sample Preparation

Urine Samples

Three milliliters of each thawed urine sample was pipetted into centrifuge tubes containing 30 μ l of the 2 μ g/ml internal standard meclofenamic acid working solution and vortex-mixed for 30 seconds. Samples were incubated with 2 ml of 1.0 M acetate buffer (pH 5) and 1 ml of fresh β -glucuronidase solution for 2 hours in a 60°C water bath. The hydrolyzed samples underwent solid-phase extraction on octyl-benzyl sulfonic acid columns (United Chemical Technologies, Bristol, PA) that had been preconditioned by successive washings of methanol, water, and 0.1 M phosphate buffer (pH 6). Loaded columns were rinsed with water, 1.0 M acetic acid, and hexane and allowed to dry under moderate vacuum.

The elimination pattern of topical diclofenac is particularly relevant to veterinarians treating horses routinely subjected to drug-testing programs.

collected as previously described. Serum and urine samples were stored frozen at -20°C until analyzed by liquid chromatography-mass spectrometry (LC-MS).

Analytical Procedure for Determination of Diclofenac and 4-Hydroxydiclofenac in Test Samples—Analytical Standards

Sodium diclofenac and 4-hydroxydiclofenac (diclofenac metabolite) were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved and diluted in high-performance liquid chromatography grade methanol (99.9%) to working solution concentrations of 2 and 20 μ g/ml. Meclofenamic acid (internal standard) was obtained from Parke-Davis (Ann Arbor, MI) and likewise dissolved and diluted to a working standard concentration of 2 μ g/ml.

Analytes of interest were eluted with 3 ml of ethyl acetate-hexane solution (50-50) and evaporated to dryness under nitrogen in a 60°C water bath. The residue was reconstituted in 250 μ l of methanol and transferred to chromatography vials. All test, calibration, and positive control samples were extracted and analyzed in duplicate. Drug-free urine was fortified to concentrations of 4, 8, 12, 16, 20, 50, 100, 400, and 800 ng/ml (calibration samples) and 5, 14, 30, and 700 ng/ml (positive control samples).

The limit of quantitation (LOQ) of the urine assay was 4.0 to 8.0 ng/ml. The LOQ was determined to be the positive control sample with the lowest concentration for which theoretic and experimental concentration values differed by less than \pm 20% and for which duplicate preparations resulted in reported

concentrations with less than $\pm 20\%$ variation. In addition, nonquantitating diclofenac and 4-hydroxydiclofenac ions demonstrated signal: noise ratios of greater than 3:1. Calibration and positive control samples were prepared and analyzed concurrently with the analysis of the administration samples and were considered acceptable if all positive controls demonstrated both accuracy and precision consistent with the $\pm 20\%$ criteria.

Serum Samples

One-milliliter aliquots of each thawed serum sample were pipetted into centrifuge tubes; 2 μ l of phosphate buffer (pH 2), 5 ml of dichloromethane, and 2 μ l of 2 ng/ μ l meclofenamic acid solution were added to each tube. The tubes were capped, mixed end-over-end for 10 minutes, and centrifuged at 3,100 rpm for 10 minutes. The organic layer was transferred to conical tubes and evaporated to dryness under nitrogen at 60°C; the resulting residue was re-

Waters 2695 Separations Module (Waters Corporation, Milford, MA) run in positive electrospray ionization mode with MassLynx v3.5 acquisition software (MicroMass). Chromatographic separation was accomplished on an Atlantis dC₁₈, 3 μ m, 2.1 \times 150 mm column (Waters Corporation) with 0.1% (v/v) formic acid in Milli-Q (Millipore, Billerica, MA) water (component A) and 0.1% (v/v) formic acid in acetonitrile (component B). Flow rate was 0.25 ml/min, and column temperature was held at 35°C. The gradient consisted of an increase from 30% component B to 70% component B over a 3-minute period followed by a 6-minute increase to 90% component B.

Serum samples were analyzed for diclofenac only, whereas urine samples were analyzed for both diclofenac and 4-hydroxydiclofenac. Analytes of interest and respective retention times were 4-hydroxydiclofenac at 7.4 minutes and diclofenac at 8.8 minutes, with internal standard meclofenamic acid detected at 9.7 min-

The highest urine concentrations of diclofenac occurred 6 hours after the final application of the cream.

constituted in 50 μ l of methanol. The tubes were vortex-mixed for 30 seconds, and the contents were transferred to chromatography vials. All test, calibration, and positive control samples were extracted and analyzed in duplicate. Drug-free serum was fortified to concentrations of 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 10, 12, 16, 20, 30, and 50 ng/ml (calibration samples) and 0.2, 0.4, 0.8, 1.0, 4.0, 12, and 30 ng/ml (positive control samples). The LOQ of the serum assay was 0.2 to 0.8 ng/ml.

Sample Analysis

LC-MS data were obtained on a MicroMass Quattro Micro (Cary, NC) in tandem with a

For compound identification and quantification purposes, a single transition of the ion at mass:charge ratio 296 to 278 (m/z 296>278) was selected for meclofenamic acid. Two transitions were utilized for 4-hydroxydiclofenac: m/z 312>266 for quantification and m/z 312>231 for compound verification purposes. Three transitions were utilized for diclofenac analysis: m/z 296>215 for quantification and m/z 296>250 and m/z 296>278 for compound identity verification purposes. The peak area ratios for diclofenac and 4-hydroxydiclofenac from each test sample, calibrator, and positive control urine samples were calculated by dividing the area of the ions at

TABLE 1. Urine Diclofenac Concentration (ng/ml; Mean \pm SD) versus Time after the Final Topical Administration of Three Different Doses of Diclofenac Liposomal Cream Twice Daily for 10 Days

<i>Time after Dosing (hr)</i>	<i>1\times Label Dose</i>	<i>2\times Label Dose</i>	<i>4\times Label Dose</i>
0.25	103.6 \pm 119.4	247.5 \pm 283.5	411.3 \pm 236.7
6	256.2 \pm 96.8	283.6 \pm 153.3	502.1 \pm 192.0
12	99.5 \pm 50.4	153.4 \pm 99.2	301.6 \pm 173.7
24	56.5 \pm 33.0	97.0 \pm 73.5	179.6 \pm 75.1
48	23.6 \pm 9.1	76.3 \pm 31.8	157.9 \pm 81.4
72	12.2 \pm 10.0	64.1 \pm 38.7	121.2 \pm 35.3

TABLE 2. Urine 4-Hydroxydiclofenac Concentration (ng/ml; Mean \pm SD) versus Time after the Final Topical Administration of Three Different Doses of Diclofenac Liposomal Cream Twice Daily for 10 Days

<i>Time after Dosing (hr)</i>	<i>1\times Label Dose</i>	<i>2\times Label Dose</i>	<i>4\times Label Dose</i>
0.25	72.9 \pm 34.24	178.5 \pm 84.1	263.6 \pm 154.7
6	119.3 \pm 49.7	173.5 \pm 51.7	273.4 \pm 195.07
12	79.5 \pm 36.8	161.5 \pm 28.7	259.3 \pm 64.1
24	43.1 \pm 22.8	84.6 \pm 59.4	166.9 \pm 13.1
48	20.5 \pm 8.1	46.5 \pm 14.5	111.7 \pm 41.1
72	14.1 \pm 5.8	53.1 \pm 20.1	102.3 \pm 30.0

m/z 215 and 266, respectively, at the retention times of diclofenac and 4-hydroxydiclofenac by the area of the ions at m/z 278 at the retention time of meclofenamic acid. The resulting peak area ratios versus the concentrations of the corresponding calibrators were plotted, and the calibration line was determined by linear nonweighted regression. A correlation coefficient of at least 0.999 was deemed acceptable. Concentrations of diclofenac and 4-hydroxydiclofenac in test and control samples were determined from the slope and intercept of the corresponding regression equation.

RESULTS

After 10 days of topical administration of

1% diclofenac liposomal cream at 1 \times , 2 \times , and 4 \times the recommended label dose, diclofenac and 4-hydroxydiclofenac were present in all urine samples collected from 0.25 to 72 hours after the final application (Tables 1 and 2). As shown in Figures 1 and 2, the highest urine concentrations of diclofenac and 4-hydroxydiclofenac occurred 6 hours after the final application of the cream at both 1 \times and 4 \times the label dose. For example, following application of 1 \times the label dose twice daily for 10 days, the highest urine concentrations occurred 6 hours after the last dose was applied, with mean peak concentrations of diclofenac and 4-hydroxydiclofenac of 256.2 \pm 92.9 and 119.6 \pm 49.7 ng/ml, respectively (mean \pm SD). After appli-

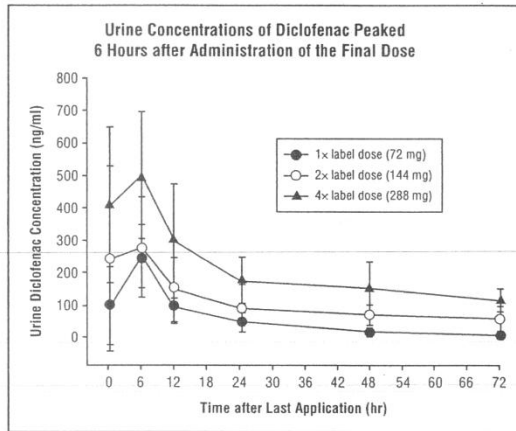


Figure 1. Urine diclofenac concentration (mean \pm SD) versus time from six horses after the final topical administration of three different doses of diclofenac liposomal cream twice daily for 10 days.

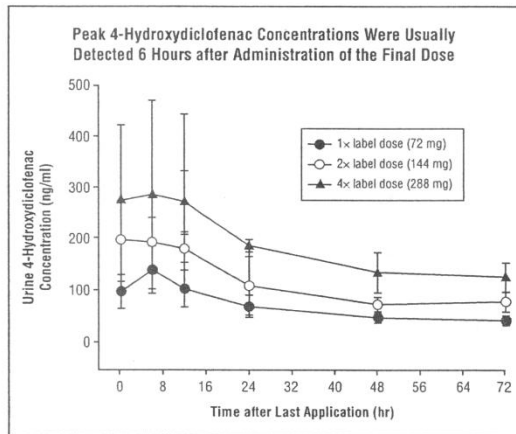


Figure 2. Urine 4-hydroxydiclofenac concentration (mean \pm SD) versus time from six horses after the final topical administration of three different doses of diclofenac liposomal cream twice daily for 10 days.

cation of 2 \times the label dose, the highest mean concentration of diclofenac also occurred in the samples collected 6 hours after the final application, but 4-hydroxydiclofenac concentrations were higher in the samples collected 0.25 hours after the final application (Figures 1 and 2). After administration of 1 \times , 2 \times , and 4 \times the label dose twice daily for 10 days, the decline in diclofenac and 4-hydroxydiclofenac concentrations occurred gradually over the next 3 days, with both the parent compound and the metabolite present in concentrations above the LOQ of the assay (4.0 to 8.0 ng/ml) 72 hours after the last application of each dosage regimen.

After 10 days of topical administration of 1% diclofenac liposomal cream at 1 \times , 2 \times , and 4 \times the recommended label dose, diclofenac was present in all serum samples collected from 0.25 to 48 hours after the final application (Figure 3 and Table 3). Similar to the results of the urine analysis, peak diclofenac concentrations were usually detected in the samples collected 6 hours after the final application. Mean concentrations of diclofenac in serum samples collected 0.25 and 6 hours after the final application of the 2 \times label dose, however, were essentially the same at 3.8 \pm 1.7 and 3.8 \pm 1.2 ng/ml, respectively. After 6 hours, serum

concentrations of diclofenac declined slowly and remained above the LOQ of the assay (0.2 to 0.8 ng/ml) for 48 hours after all dosing regimens and for 72 hours after application of 2× and 4× the recommended label dose.

DISCUSSION

The diclofenac liposomal formulation used in this study is unique in equine medicine. Liposomes are microscopic vesicles composed of membrane-like lipid layers surrounding an inner compartment.⁸ The layers are composed of phospholipids, which are lipophilic at one end and hydrophilic at the other. Liposomal preparations are often used to transport hydrophilic compounds across membranes. The compounds are dissolved in the aqueous phase of the liposome, which is located between the lipid layers and in the central core. Although effective, the amount of a hydrophilic drug that can be delivered by this method is generally limited. However, because diclofenac is

highly lipophilic, a relatively large amount of the drug will also dissolve in the lipid portion of the liposome. This maximizes the amount of drug that can be transported across cell membranes and delivered to the site of inflammation when the preparation is applied topically.

The term *locally enhanced topical delivery* (LETD) has been used to describe this local ac-

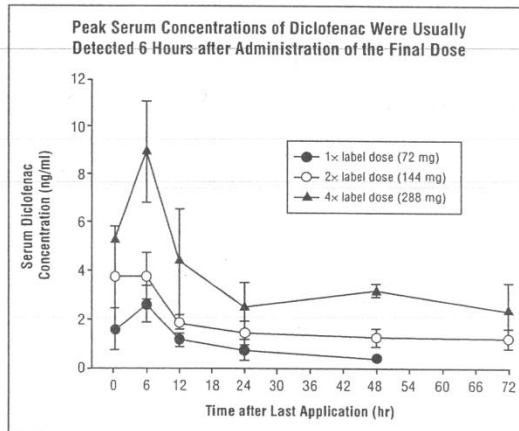


Figure 3. Serum diclofenac concentration (mean ± SD) versus time from six horses after the final topical administration of three different doses of diclofenac liposomal cream twice daily for 10 days.

TABLE 3. Serum Diclofenac Concentration (ng/ml; Mean ± SD) versus Time after the Final Topical Administration of Three Different Doses of Diclofenac Liposomal Cream Twice Daily for 10 Days

Time after Dosing (hr)	1× Label Dose	2× Label Dose	4× Label Dose
0.25	1.6 ± 0.9	3.8 ± 2.1	5.3 ± 1.6
6	2.6 ± 0.8	3.8 ± 1.0	9.1 ± 2.2
12	1.1 ± 0.2	1.9 ± 0.3	4.5 ± 1.0
24	0.8 ± 0.4	1.5 ± 0.5	2.5 ± 0.3
48	0.4 ± 0.0	1.3 ± 0.4	3.3 ± 0.3
72	<LOQ	1.2 ± 0.4	2.4 ± 1.1

cumulation of drug into a target tissue.⁹ LETD was demonstrated in a recent study of seven horses with subcutaneously implanted tissue cages, which revealed that the highest mean concentration of diclofenac (76.2 ± 29 ng/ml) was detectable in the carrageenan-induced inflammatory transudate within 6 to 18 hours after topical application of diclofenac liposomal cream.¹⁰ In addition, this transudate had lower prostaglandin E₂ concentrations than the transudate from placebo-treated tissue cages.

Compared with other conventional creams and ointments, liposomal formulations provide better penetration and sustained release of therapeutic agents.¹¹ The results of this study are consistent with these previous reports. For example, the highest concentrations of diclofenac in urine and serum were generally detected 6 hours after administration. In addition, urine and serum concentrations of diclofenac declined slowly over 2 to 3 days after the last application. These findings indicate that the liposomal formulation of diclofenac is slowly absorbed following topical application, which is consistent with the prolonged effect

Urine and serum concentrations of diclofenac declined slowly over 2 to 3 days after the last application.

observed in the tissue cage inflammatory study.¹⁰

Systemic administration of NSAIDs can be associated with side effects that include gastrointestinal ulceration and renal toxicity. The results of this study indicate that topical application of diclofenac in a liposomal formulation is unlikely to result in serum concentrations that would be associated with systemic toxicity. For example, even when applied at 4× the label dose twice daily for 10 days, serum concentration of diclofenac did not exceed 15 ng/ml in any of the

horses. Although the pharmacokinetics of diclofenac in horses following systemic administration has not been determined, oral administration of recommended therapeutic doses in children (1 to 2 mg/kg) resulted in peak serum concentration of 2.4 ± 1.3 µg/ml.¹² This would be consistent with concentrations of therapeutic doses of NSAIDs in horses, which are typically in the µg/ml range.¹³⁻¹⁵ Thus, serum concentrations associated with systemically active doses of NSAIDs are 100× or more than those occurring after topical application of diclofenac liposomal cream to horses.

As an NSAID, diclofenac is regulated in most medication control programs under which performance horses compete. As such, the results of this study may be useful in assisting veterinarians in determining adequate withdrawal times to avoid positive postcompetition tests. For example, the United States Equestrian Federation (USEF) allows diclofenac to be present in serum collected from horses competing under their Therapeutic Substance Rule as long as the concentration does not exceed 5 ng/ml.¹⁶ To avoid exceeding this maximum per-

mitted serum concentration, veterinarians should prescribe a 5-inch ribbon of Surpass to be applied to no more than one affected site every 12 hours.¹⁷ In addition, administration of diclofenac cream should be discontinued at least 12 hours before the horse competes in a USEF-sanctioned event or show.¹⁷

In contrast, under the USEF's No Foreign Substance Rule, which is essentially the same as the Fédération Equestre Internationale (FEI) rule, no amount of diclofenac would be permitted to be present in a competing horse.¹⁶

Because urine concentrations of diclofenac were determined for only 72 hours after administration in this study and because the limits of detection for the testing methods used by these organizations are not known, it is diffi-

CONCLUSION

If applied correctly, diclofenac liposomal suspension is a safe and effective method to treat focal sites of inflammation in horses. Because of the large variation in drug-testing methodolo-

The USEF allows diclofenac to be present in serum as long as the concentration does not exceed 5 ng/ml.

cult to predict withdrawal time for these rules. A minimum of 1 week may be sufficient, but veterinarians should consult with regulatory authorities of the USEF and FEI for more specific recommendations.

In a similar manner, many different analytical methods are used in the United States to detect NSAIDs such as diclofenac in urine samples collected from horses after racing. Therefore, it is not possible to recommend a blanket withdrawal time for horses racing in different jurisdictions. Nevertheless, some generalities can be made. If the jurisdiction uses a thin layer chromatography-based method to screen for diclofenac, at least 24 hours or the mandatory withdrawal period for that jurisdiction would be recommended when one site is being treated with diclofenac cream. If two or four sites on a horse are being treated, the withdrawal time should be increased to a minimum of 48 or 72 hours, respectively. These withdrawal time estimates are based on typical limits of detection for NSAIDs using thin layer chromatography, which range from 200 to 500 ng/ml. If the jurisdiction is testing for diclofenac using an instrument-based method, such as the LC-MS method described in this study, or an immunoassay, a minimum withdrawal time of 96 hours or 1 week, respectively, should be followed because of the increased sensitivity of these methods compared with thin layer chromatography.

gies used by laboratories carrying out testing for show and racing authorities, withdrawal guidelines vary from a minimum of 24 hours to at least 1 week. Veterinarians, horse owners, and trainers should consult with the regulatory authorities and racing laboratories in their jurisdiction for additional guidance.

ACKNOWLEDGMENT

The authors thank medical writer Tad B. Coles, DVM, of Overland Park, KS, for technical assistance in writing and editing this manuscript.

REFERENCES

1. Kallings P: Nonsteroidal anti-inflammatory drugs. *Vet Clin North Am Equine Pract* 9(3):523-541, 1993.
2. Heyneman CA, Lawless-Liday C, Wall GC: Oral versus topical NSAIDs in rheumatic diseases: A comparison. *Drugs* 60(3):555-574, 2000.
3. Roth SH, Shainhouse JZ: Efficacy and safety of a topical diclofenac solution (pennsaid) in the treatment of primary osteoarthritis of the knee: A randomized, double-blind, vehicle-controlled clinical trial. *Arch Intern Med* 164(18):2017-2023, 2004.
4. Scholer DW, Ku EC, Boettcher I, Schweizer A: Pharmacology of diclofenac sodium. *Am J Med* 80(4B):34-38, 1986.
5. Brogden RN, Heel RC, Pakes GE, et al: Diclofenac sodium: A review of its pharmacological properties and therapeutic use in rheumatic diseases and pain of varying origin. *Drugs* 20(1):24-48, 1980.
6. Todd PA, Sorkin EM: Diclofenac sodium. A reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. *Drugs* 35(3):244-285, 1988.
7. Lynn RC, Hepler DI, Kelch WJ, et al: Double-blind-

- ed placebo-controlled clinical field trial to evaluate the safety and efficacy of topically applied 1% diclofenac liposomal cream for the relief of lameness in horses. *Vet Ther* 5(2):128–138, 2004.
8. Bertone JJ, Lynn RC, Vatistas NJ, et al: Clinical field trial to evaluate the efficacy of topically applied diclofenac liposomal cream for the relief of joint lameness in horses. *Proc AAEP* 48:190–193, 2002.
 9. Grahame R: Transdermal non-steroidal anti-inflammatory agents. *Br J Clin Pract* 49(1):33–35, 1995.
 10. Caldwell FJ, Mueller PO, Lynn RC, Budsberg SC: Effect of topical application of diclofenac liposomal suspension on experimentally induced subcutaneous inflammation in horses. *Am J Vet Res* 65(3):271–276, 2004.
 11. Vemuri S, Rhodes CT: Preparation and characterization of liposomes as therapeutic delivery systems: A review. *Pharm Acta Helv* 70(2):95–111, 1995.
 12. Romsing J, Ostergaard D, Senderovitz T, et al: Pharmacokinetics of oral diclofenac and acetaminophen in children after surgery. *Paediatr Anaesth* 11(2):205–213, 2001.
 13. Gerring EL, Lees P, Taylor JB: Pharmacokinetics of phenylbutazone and its metabolites in the horse. *Equine Vet J* 13(3):152–157, 1981.
 14. Lees P, Landoni MF: Pharmacodynamics and enantioselective pharmacokinetics of racemic carprofen in the horse. *J Vet Pharmacol Ther* 25(6):433–448, 2002.
 15. Dyke TM, Sams RA, Thompson KG, Ashcraft SM: Pharmacokinetics of multiple-dose administration of eltenac in horses. *Am J Vet Res* 59(11):1447–1450, 1998.
 16. United States Equestrian Foundation: 2005 United States Equestrian Foundation, Inc. Rule Book. Available at www.usef.org/content/rules/ruleBook/2005; accessed March 22, 2005.
 17. United States Equestrian Foundation: Practical Advice Regarding The 2005 Equine Drugs & Medications Rule. Available at www.usef.org/content/drugsMeds; accessed March 22, 2005.

Lynn Hovda

From: Lynn Hovda [Lynn.Hovda@pop3.state.mn.us]
Sent: Thursday, March 10, 2011 11:11 AM
To: 'Richard.Krueger@state.mn.us'; Mary Manney (Mary.Manney@state.mn.us)
Subject: FW: FW: Surpass

Just wanted to resend this email from Dr. Cole, who did the original research on Surpass. She recommends 0.1 ng/ml and we compromised with the HBPA on 2 ng/ml (they recommended 5 ng/ml). Lynn

From: Lynn Hovda [mailto:lhovda@safetycall.com]
Sent: Monday, January 10, 2011 12:41 PM
To: 'mary.manney@state.mn.us'
Cc: 'lynn.hovda@state.mn.us'
Subject: FW: FW: Surpass

Hoping I remembered to send this to you. Lynn

From: Cynthia Cole [mailto:cynthia.cole2010@gmail.com]
Sent: Wednesday, January 05, 2011 2:18 PM
To: Lynn Hovda
Subject: Re: FW: Surpass

Hi Lynn,
No problem, contact me any time with horse questions. I am lost in the world of dogs and cats.

I left Novartis in the middle of November and am now with a very small start company called Piedmont Pharmaceuticals. It is some of the same folks who started Blue Ridge Pharmaceuticals some time ago. It is so nice to be back in a small company. Novartis was fine, but big pharma development is tedious at best.

I am basing my recommendations on Table 3 results and the fact that Surpass is approved for a BID administration. One could assume and I think in this case that is a safe assumption, that the pharmacological effects are waning at 12 hours, and probably gone by 48 hours. Remember there are those out there that don't think this stuff works at all. So I would recommend a 48 hour withdrawal for a single leg treated and I would set a 1 ng/ml threshold. 2ng/ml and 5 ng/ml or too high based on the concentrations achieved with the 1x dose. You could make a case that 1 ng/ml is too high as well, because some of the 1x horses were below that at 12 hours post treatment, so maybe 0.5 ng/ml would be better. However, if you set a threshold you need the lab to be able to consistently and reliably quantitate at the concentration and 0.5 ng/ml might be pushing the limit. In addition, the mean at 48 hours 1x dose was 0.4 ng/ml, so 0.5 is awful close. I never want to catch some one who followed the rules. If I catch someone I want to be really really sure that they didn't follow the rule and I think 1.0 ng/ml at 48 hours would be a defensible number and a reasonable withdrawal time. I am probably bias but of all the things they could be treating these horses with Surpass is one I am least concerned with so I don't really think a long withdrawal time would be in the best interest of the horse. 1 ng/ml might seem a little close to the 0.4 ng/ml mean but remember this was applied 2x a day for 10 days to one joint and I doubt very few people actually do that. Finally, all of the means for the 2x and 4x treatments are above 1 ng/ml for the time period we collected, so it would be hard for someone to treat 2 limbs with in 48 hours and not be above the limit.

So that is my thought process for what it is worth. Hope it is helpful and let me know what you think.

Hope all is well with you!

Cindy

On Wed, Jan 5, 2011 at 2:49 PM, Lynn Hovda <lhovda@safetycall.com> wrote:

Your Novartis email bounced back at me so Libby Biggs gave me this one. Do you have another address as well? Lynn

From: Lynn Hovda
Sent: Wednesday, January 05, 2011 1:47 PM
To: 'cynthia.cole@novartis.com'
Subject: Surpass

Cindy:

I am really sorry to bother you again about the IDEXX study, but the Minnesota Racing Commission is trying to set a regulatory limit with detection times for Surpass ointment. We currently use LC/MS in serum or plasma as our primary means of testing.

Based on the study, it appears that the LOQ is 0.8 ng/ml with a 96 hour detection time in serum for 1 joint. We don't necessarily have to use an LOQ but I do need to tell the commission that there are **no** pharmacological effects at whatever detection time is chosen.

Dr. Tobin and the HBPA are suggesting 5 ng/ml at 48 hours with no mention of pharmacological effects. He later suggested 2 ng/ml at 48 hours.

Can you provide any assistance here?

Lynn

Lynn R. Hovda, RPH, DVM, MS, DACVIM

Director of Veterinary Services

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From: Lynn Hovda [mailto:Lynn.Hovda@state.mn.us]
Sent: Thursday, April 14, 2011 11:10 AM
To: Tobin, Thomas
Subject: Diclofenac Warning

Tom: I am looking in the HBPA book and do not see a horsemen's warning for this. Can you provide one or direct me to the specific area in the book so I can add it to the info for the veterinarian and trainer's manuals? Many thanks, Lynn

OK, DICLOFENAC??? I believe we compromised on a serum level of 2 ng/ml at 48 hours and I am happy.

2 ng/ml in serum, and specify the manufacturers label dose and 48 hours out. And note the Horsemen's warning in the HBPA book.

*Lynn R. Hovda, RPH, DVM, MS, ACVIM
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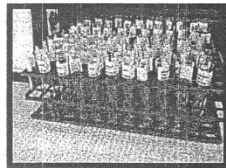
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RMTC Makes Withdrawal Time Recommendations, Announces Progress of its Drug Testing Initiatives Task Force



Based on the recommendation of its Scientific Advisory Committee, the Racing Medication and Testing

Consortium (RMTC) board of directors has recommended scientifically identified withdrawal guidelines and plasma thresholds for the short-acting bronchodilator glycopyrrolate, the muscle relaxant methocarbamol, and four anabolic steroids, three of which are endogenous (naturally occurring) in the horse. A recommendation for the non-steroidal anti-inflammatory medication firocoxib has also been approved pending resolution of one outstanding issue.

"Administration studies on these medications were developed and funded by the RMTC over the past few years in an effort to provide horsemen and practicing veterinarians with withdrawal guidelines that will enable the therapeutic use of these medications without compromising the integrity of racing competition or the welfare of our human and equine athletes," said Dr. Rick Arthur, chair of the RMTC Scientific Advisory Committee and equine medical director for the California Horse Racing Board.

"The results of these studies have been eagerly anticipated by the industry, and we will continue to conduct important research on other therapeutic medications that are commonly used in managing the health of racehorses."

The board made these recommendations at its meeting at the University of California-Davis on October 12, 2010.

The specific recommendations are as follows:

- ✓ Glycopyrrolate: Threshold concentration of 3.5 pg/ml in serum or plasma, which regulates a 24-hour withdrawal guideline for a single, intravenous total dose of 1 mg.
- ✓ Methocarbamol: Threshold concentration of 1 ng/ml, which regulates a 48-hour withdrawal guideline for a single intravenous dose of 15 mg/kg.
- ✓ Firocoxib: Threshold concentration of 40 ng/ml in serum or plasma, which regulates a 7-day withdrawal guideline when administered at the FDA-approved label dose, pending a review of clinical effects by the RMTC Scientific Advisory Committee.
- ◆ Boldenone: Screening limit no greater than 100 pg/ml in serum or plasma with a confirmatory threshold no greater than 25 pg/ml for all horses regardless of sex, which regulates an 82-day withdrawal time.
- ◆ Nandrolone: Screening limit no greater than 100 pg/ml in serum or plasma with a confirmatory threshold no greater than 25 pg/ml for geldings, fillies and mares, which regulates a 35-day withdrawal time. Male horses other than geldings will not be tested for nandrolone in blood.
- ◆ Stanozolol: Screening limit no greater than 100 pg/ml in serum or plasma with a confirmatory threshold no greater than 25 pg/ml for all horses regardless of sex, which regulates a 47-day withdrawal time.
- ◆ Testosterone: Screening limit no greater than 100 pg/ml in serum or plasma with a confirmatory threshold no greater than 25 pg/ml for geldings, fillies and mares and a confirmatory limit of 2 mcg/ml for male horses other than geldings, which regulates a 30-day withdrawal time.

Meeting business is continued on page 2.

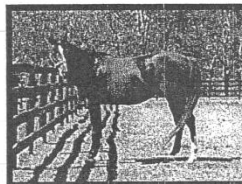
Continued from Page 1

The RMTC board expects to be able to issue recommendations for acepromazine, pyrilamine, mepivacaine, lidocaine, procaine and butorphanol at its next full meeting.

In addition, the RMTC board of directors approved a plasma threshold of 10 mcg/ml for DMSO, which would allow for the use of DMSO as a topical leg paint but would not allow for the oral or intravenous administration of the drug. The board also recommended that DMSO be reclassified by the RCI Drug Testing Standards and Practices Committee to a Class 4 substance when appearing in samples in excess of the threshold concentration.

Based on a report provided by the Scientific Advisory Committee, the RMTC board expects to be able to issue recommendations for acepromazine, pyrilamine, mepivacaine, lidocaine, procaine and butorphanol at its next full meeting in March 2011.

Also at the meeting, RMTC Executive Director Dr. Scott Waterman provided an update on the progress of the RMTC Drug Testing Initiatives Task Force, which has been working toward drug testing quality assurance and laboratory accreditation since its inception in 2008. He noted that before the task force was initiated, only five of the 18 laboratories conducting drug testing on racehorses were accredited to ISO 17025 standards. Dr. Waterman predicted that number will increase to 11 out of 19 laboratories by the end of 2011. In addition, three additional laboratories have expressed an interest in becoming RMTC-accredited following RMTC Vice Chairman Alan Foreman's remarks at The Jockey Club Round Table Conference in August.



"We are thrilled with the commissions' and their laboratories' response to the challenge laid out by Mr. Foreman as part of his remarks at The Jockey Club Round Table Conference," said Dr. Waterman. "The successful implementation of this program will improve industry confidence in its laboratory infrastructure and put our labs on par with any in the world."

In other RMTC business:

- ◆ Board members received updates on funded research for several potential doping agents and a progress report on research on corticosteroids.
- ◆ Dr. Heather Knych, who was funded by the RMTC post-doctoral research fellowship program, presented a summary of her ongoing corticosteroids research.
- ◆ The RMTC will develop a penalty structure for the 2ug/ml threshold limit for phenylbutazone

to recommend to the RCI for their consideration at the next RMTC board meeting.

- ◆ Meeting attendees were given a guided tour of the Kenneth L. Maddy Equine Analytical Chemistry Laboratory and a demonstration of the equine treadmill at the UC-Davis Veterinary Medical Teaching Hospital.

"I would like to thank Dr. Scott Stanley and everyone at the Ken Maddy laboratory who helped host our group at this incredible facility," said RMTC Chairman Dr. Robert Lewis. "We truly appreciated their hospitality and willingness to provide our board members with a unique learning experience."

For more information on AAEP white papers, visit aaep.org. Copies can also be found on the RMTC website—rmcnet.com

AAEP Releases Standardbred Welfare Recommendations

The American Association of Equine Practitioners (AAEP) Racing Committee has modified recommendations that the group previously developed for Thoroughbreds and Quarter Horses for the harness racing industry. The white paper, "Putting the Horse First: Veterinary Recommendations for the Safety and Welfare of the Standardbred Racehorse," provides guidelines specific to the breed that are concentrated in four main areas: the racing business model, the owner-trainer-veterinarian relationship, medication, and the public perception of racing.

The recommendations are as follows: standardization and enhancement of pre-race and post-race veterinary examinations across the 17 U.S. jurisdictions that hold harness racing; consistent participation by Standardbred tracks in a national injury reporting system; adoption of best practice procedures that emphasize safety of the racing surface; increased racetrack security to ensure compliance by all racing participants with medication rules; and a modified structure for claiming races, which allows claims to be rescinded if the post-race drug test is positive (horses that do not finish or sustain a catastrophic injury during the race or while leaving the track should remain the property of the original owner unless the individual with the claim decides to accept the horse).

Excerpt from Alan Foreman's Report to The Jockey Club Round Table

We encourage all of the laboratories who perform drug testing for racing in this country to commit to accreditation to the RMTC Standards. These standards are so important that at a minimum, beginning in 2012, we should not allow drug testing of our Triple Crown, Breeders' Cup and graded stakes races to be performed at any laboratory not accredited to the RMTC Standards. Indeed, for the sake of our sport, each and every one of you should demand that the drug testing for all of our races, regardless of when and where, be tested by a laboratory accredited to the RMTC accreditation standards. Our regulators need to take the necessary steps in their contracts for testing services to require that the laboratory they engage be accredited to the RMTC Standards. For those who say we could never get this done because there was no way to pay for it, let me tell you about leadership by example. The New York Thoroughbred Horsemen's Association, the owners and trainers who race in New York, is providing the funds necessary for New York's new laboratory to become accredited to the RMTC Standards, and The Jockey Club, demonstrating its ongoing commitment to this effort, will provide financial assistance to any laboratory seeking RMTC accreditation.

It is one thing to achieve accredited status. It then becomes important to ensure that the laboratory is able, on a continuing basis, to accurately detect drugs at concentrations relevant to our sport and with results that are correct. This is accomplished through quality assurance and proficiency testing. Through blind and double-blind testing, the assessor is able to determine whether a laboratory can detect a drug and whether its results are correct. Quality assurance and laboratory proficiency go hand-in-hand with accreditation. In our racing industry, quality assurance and laboratory proficiency have historically been divided between two separate organizations of our scientists, each of whom tested themselves and with no collaboration between the programs. Following up on the recommendations of the [RMTC Drug Testing Initiatives Task Force], these two programs are now being merged into a single new Equine Quality Assurance Program developed by the RMTC, but which will be administered and monitored by an independent third party. This is a totally new concept for our industry and another major step forward.

Consolidation of our existing 18-laboratory infrastructure, and creating one or more industry-sponsored research and reference laboratories, while absolutely essential for the future of our industry, is, and was always deemed to be, the most ambitious and difficult recommendation to achieve. There are political and funding issues that we knew would limit short-term progress and that was to be expected. Without further comment, I can tell you that I believe significant consolidation of our current laboratory structure is already under way at this time, and you will be hearing about this consolidation in the coming months.

As to the recommendation for one or more industry-sponsored research and reference laboratories, four of the laboratories seeking RMTC accreditation — New York, California, Pennsylvania and Kentucky — are, or will become, recognized research and reference laboratories, the kind envisioned when we made these recommendations. These are all state-of-the-art programs that combine drug testing and research. They will collaborate on all of our testing and research issues, an unprecedented and monumental step for our industry. They will help us answer the questions that continually arise about drug testing in racing and make it possible for us to deliver a coherent explanation. More importantly for this moment, two of our laboratories are presently collaborating on the identification and confirmation of certain gene and blood-doping agents, the new generation of drugs that I warned of two years ago, that are now finding their way into the equine sport. Funding for this effort is being made possible by the RMTC. I am confident that you will be hearing about the fruits of their labor in the not too distant future. For a full transcript of this and other reports at the 2010 Round Table Conference, please visit jockeyclub.com.

"Consolidation of our existing 18-laboratory infrastructure, and creating one or more industry-sponsored research and reference laboratories, while absolutely essential for the future of our industry, is, and was always deemed to be, the most ambitious and difficult recommendation to achieve."

-Alan Foreman

Upcoming Industry Meetings and Conferences

- ◆ AAEP 56th Annual Convention: December 4-8
- ◆ University of Arizona RTIP Symposium on Racing & Gaming: December 6-9
- ◆ Dr. Waterman's presentation to the Kentucky Thoroughbred Farm Managers' Club: March 2
- ◆ National HBPA Winter Convention: March 17-21
- ◆ RCI Annual Conference: March 23-26



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UPDATE:
The RCI board of
directors approved
the RMTC
phenylbutazone
recommendation that
decreases the
threshold for that
medication from
5 mcg/ml to 2 mcg/ml.

Our Mission

The RMTC strives to develop and promote uniform rules, policies and testing standards at the national level; coordinate research and educational programs that seek to ensure the integrity of racing and the health and welfare of racehorses and participants; and protect the interests of the racing public.



Industry News

AAEP Develops Guidelines on Racehorse Care

The American Association of Equine Practitioners (AAEP) recently developed the white paper, "Clinical Guidelines for Veterinarians Practicing in a Pari-Mutuel Environment," to provide guidance for veterinarians treating racehorses. The guidelines encourage medical practices that place proper emphasis on the health, safety and welfare of the racehorse. Its main focus is the practice of treating a horse based on the entry date for its next race. The white paper expresses that to ensure a horse is recovered before it races, treatment should be based on a veterinary diagnosis with appropriate time allowed for a follow-up evaluation.

"Our purpose is to reinforce sound medical principles within the unique environment of racing," said AAEP President Dr. Nat White. "As horse racing continues its pursuit of increased safety, we understand the need for us, as veterinarians, to examine our role in medication usage and medical treatment. These guidelines affirm the already excellent medical care provided by many veterinarians."



The use of therapies such as shockwave therapy and hyperbaric oxygen therapy are also addressed, along with recommendations on maintaining medical records and changing the current racetrack veterinary business model to one that bases billing on professional services rendered, rather than the dispensing or administration of medication.

To view the full paper, go to www.aaep.org/white_papers.htm.

Breeders' Cup Passes Drug Ban Rule for Trainers

In an effort to increase international participation in the Breeders' Cup World Championships, the Breeders' Cup board of directors approved a new international nominations program and extended its ban on trainers with medication infractions to include Class 1 substances in any racing jurisdiction in the year prior to the event.

Class 1 drugs are stimulants and depressants that have the highest potential to affect the outcome of a race and have no accepted medical use in horses, according to the Association of Racing Commissioners International's guidelines. The vote was passed unanimously and will go into effect in 2011.

Kentucky Approves Out-of-Competition Testing

The Kentucky Horse Racing Commission enabled the state to meet Breeders' Cup World Championships standards by approving out-of-competition testing as an emergency regulation, immediately in effect.

Ned Bonnie, who headed the joint committee that developed the rule, believed it was necessary that it be passed as an emergency regulation due to concerns about equine welfare and safety, and for the protection of the betting public.

Out-of-competition testing targets blood-doping substances such as erythropoietin (EPO) that fail to be detected in post-race testing.

The commission recommended a five- to 10-year license suspension for the owner and/or trainer of the horse, a fine of up to \$50,000 and a forfeiture of purse money.

2011, 34(6): 605-608
MEZO
Pharmacokinetics of glycopyrrolate following intravenous administration
in the horse

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(Paper received 7 September 2010; accepted for publication 16 December 2010)

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Glycopyrrolate, designated a class 3 substance by the Association of Racing Commissioners International, Inc., is regulated in racing horses because of its potential to affect performance. Although it has veterinary clinical applications by inhibiting parasympathetic activity, its use near race day is prohibited and positive reports from posttrace samples in the US are relatively common. Accordingly, the American Association of Equine Practitioners identified glycopyrrolate as a therapeutic substance used by race track practitioners for legitimate therapeutic purposes, and the Racing Medication and Testing Consortium (RMTC) has requested studies of the disposition of glycopyrrolate as part of its efforts to acquire reliable data upon which to propose thresholds and withdrawal time recommendations for therapeutic substances used in racing horses.

Glycopyrrolate, a quaternary ammonium salt and synthetic anti-cholinergic drug, exerts peripheral anti-muscarinic effects on the respiratory tract without imparting substantial effects on the central nervous system (CNS) compared to other muscarinic antagonists such as atropine. Glycopyrrolate differs from these other muscarinic antagonists because it penetrates the CNS poorly due to its highly polar quaternary ammonium group and its permanent ionization at physiological pH compared to its more lipophilic congeners.

Previous studies have investigated glycopyrrolate pharmacokinetics in humans to a limited extent (Pentilla *et al.*, 2001). However, to our knowledge, pharmacokinetic studies of this drug in the horse have not been reported likely due to limitations in sensitivity of the methods that are commonly used. Quantitative methods with limits of detection and quantification well below those of previously reported methods have recently been developed and validated through the RMTC research program. These validated methods (Rumpler *et al.*, 2010b) demonstrate necessary sensitivity, accuracy, and precision to measure plasma concentrations sufficient to perform pharmacokinetic analysis through the 24-h time period after administration of clinically relevant doses to horses. Such investigations could contribute to the RMTC effort to establish a plasma threshold and to recommend a withdrawal time for this drug in race horses. Therefore, this study investigated the disposition of glycopyrro-

late following intravenous administration of a 1-mg dose in the horse.

Eight, healthy, adult, Thoroughbred geldings, ranging in age from 5 to 10 years and weighing from 518 to 580 kg were used in these studies. All study horses were housed in grass paddocks at the University of Florida, Veterinary Medical Center (Gainesville, FL), maintained on a diet of commercially available grain mixture, and had open access to water and hay at all times. Horses were subjected to treadmill exercise (3 days/week) before and throughout the duration of these studies. The experimental protocol was approved, and facilities were inspected by the University of Florida Institutional Animal Care and Use Committee.

All horses were administered 1 mg (1.72–1.93 µg/kg) of glycopyrrolate (glycopyronium bromide, American Regent, Inc., Shirley, NY, USA) into the right jugular vein. Whole blood samples were collected from the left jugular vein via needle venipuncture into partially evacuated tubes containing lithium heparin. Blood samples were stored on ice until the plasma was concentrated by centrifugation (2500–3000 rpm or 776–1318 g) at 4 °C for 15 min. Harvesting of plasma took place within 1 h of sample collection, and 2–4-mL aliquots of plasma were immediately frozen at –20 °C and stored within 24 h at –80 °C until analyzed. Collection times included a timepoint before drug administration and 5, 10, 15, 20, 30, and 45 min and 1, 2, 3, 4, 6, 8, 24, 48, 72, 96, and 168 h after intravenous administration. Specimens were collected from two of the horses only through 24 h after dosing.

Plasma glycopyrrolate concentrations were determined using a fully validated ultra-performance liquid chromatography and tandem mass spectrometry (MS/MS) method as previously described (Rumpler *et al.*, 2010b) in accordance with US FDA recommended guidelines for bioanalytical methods. The method is characterized by a lower limit of quantitation (LLOQ) of 0.05 pg/mL of plasma.

Nonlinear least squares regression analysis was performed on plasma glycopyrrolate concentration vs. time data and pharmacokinetic parameters for all horses were estimated with both noncompartmental and compartmental analysis using Phoenix

WinNonlin® 6.1 (Pharsight, St. Louis, MO, USA). For compartmental analysis, the Gauss–Newton (Levenberg and Hartley) method was used and goodness of fit and the appropriate weighting factor were selected based on the coefficients of variation, Akaike's Information Criterion (Yamaoka *et al.*, 1978) and Schwartz's Bayesian Criterion as well as visual analysis of the graphical output (including residual plots). Secondary parameters calculated include area under the curve (AUC), terminal half-life ($t_{1/2\beta}$), apparent volumes of distribution, total plasma clearance (Cl_p), and microdistribution rate constants. For the noncompartmental analysis, the area under the plasma concentration vs. time curve (AUC_{0-24}) from time 0 to 24 h was calculated using the log-linear trapezoidal method with linear interpolation. The pharmacokinetic parameters calculated included the observed maximum plasma concentration (C_{max}), area under the plasma concentration vs. time curve to the last determined plasma concentration (AUC_l), terminal half-life ($t_{1/2}$), total plasma clearance (Cl_p), mean residence time, and steady state volume of distribution (V_{ss}). All calculations for pharmacokinetic parameters were based on methods described by Gibaldi and Perrier (1982). All pharmacokinetic parameters were calculated for each horse, and values are reported as median and range (minimum–maximum).

After intravenous administration of 1 mg of glycopyrrolate, the observed plasma concentration vs. time profile could be best described by a three-compartment model. The equation based on macro constants for this model is:

$$C_t = A \exp^{-\alpha t} + B \exp^{-\beta t} + C \exp^{-\gamma t}$$

where C_t is the plasma concentration at time (t), A, B and C are the zero time intercepts for the first, second, and third phases. Further, α , β , and γ are the exponential terms for each phase, and \exp is the base of the natural logarithm (Gabrielsson & Weiner, 2007). The weighting factor chosen with this model was $1/(Y^2)$, where Y was the observed plasma

concentration. Values for a number of pharmacokinetic variables following noncompartmental and compartmental model analysis are reported in Tables 1 and 2, respectively. Plasma glycopyrrolate concentration vs. time plots for all eight horses are depicted in Fig. 1. The drug concentrations remained above 0.5 pg/mL for all horses through 24 h after dosing (Fig 1).

To our knowledge, the pharmacokinetics of glycopyrrolate in the horse have not previously been investigated. Our data indicate that glycopyrrolate disposition in the horse exhibits triexponential decay after intravenous administration. This is characterized by an early rapid decline (Fig. 1) in plasma concentrations followed by a slow terminal phase with concentrations above the LOQ of the method for up to 168 h. All horses exhibited plasma concentrations above 1 ng/mL 5 min after drug administration followed by a precipitous decline through 20 min. Although the three-compartment model estimates for C_{max} are higher than the noncompartmental estimates because of the extrapolation back to time 0 in the compartmental model, we believe that the inclusion of these values in the model is necessary to describe the disposition of glycopyrrolate (Beaufort *et al.*, 1999). Moreover, data in humans suggest a similar pharmacokinetic profile (Pentilla *et al.*, 2001). Noncompartmental analysis provided physiologically reasonable parameter estimates. However, the volume of distribution based on the terminal phase (V_z) was unrealistically large (16.9 ± 6.7 L/kg), likely accounted for by the rapid elimination during the initial phase and low plasma glycopyrrolate concentrations during the terminal phase (Toutain & Bousquet-Melou, 2004).

Total plasma clearance is attributed to hydrolysis of glycopyrrolate and renal clearance (Rumpler *et al.*, 2010a). Although our previous studies have revealed that some glycopyrrolate is eliminated unchanged in the urine, we did not perform volumetric urine collections in this study and therefore cannot

Table 1. Pharmacokinetic parameter estimates of glycopyrrolate, determined using noncompartmental analysis, following intravenous administration of 1 mg to eight ($n = 8$) healthy adult Thoroughbred horses

Parameter	Horse								Median	Min	Max
	1	2	3	4	5	6	7	8			
λ_z (h^{-1})	0.097	0.066	0.089	0.102	0.084	0.082	0.067	0.054	0.083	0.066	0.102
$t_{1/2\lambda_z}$ (h)	7.14	10.5	7.79	6.78	8.28	8.48	10.4	12.9	8.38	6.78	12.9
C_{max} (ng/mL)	5.48	4.72	4.21	8.27	5.14	4.07	2.43	4.55	4.64	2.43	8.27
C_{last} (ng/mL) $\times 10^{-3}$	1.11	1.54	1.17	0.860	1.25	0.953	2.25	1.92	1.21	0.860	2.25
AUC_{0-24} ($h \cdot ng/mL$)	1.67	1.40	1.38	2.49	1.54	1.43	0.953	1.50	1.46	0.953	2.49
$AUC_{0-\infty}$ ($h \cdot ng/mL$)	1.68	1.42	1.40	2.50	1.55	1.44	0.987	1.53	1.49	0.987	2.50
V_z (L/kg)	12.5	19.4	14.1	7.56	13.1	14.6	27.9	22.2	14.4	7.56	27.9
Cl (mL/min/kg)	20.3	21.3	21.0	12.9	18.3	19.9	31.0	19.9	20.1	12.9	31.0
$AUMC_{0-24}$ ($h^2 \cdot ng/mL$)	1.06	0.963	1.04	0.952	0.990	0.783	1.40	1.20	1.01	0.783	1.40
MRT_{0-24} (h)	0.636	0.688	0.750	0.383	0.644	0.548	1.47	0.798	0.666	0.383	1.47
V_{ss} (L/kg)	1.05	1.68	1.35	0.383	1.08	1.00	5.13	2.10	1.22	0.383	5.12

λ_z , elimination rate constant; $t_{1/2\lambda_z}$, terminal half-life; C_{max} , observed maximum plasma glycopyrrolate concentration; C_{last} , observed plasma glycopyrrolate concentration at 24 h; AUC_{0-24} , area under the plasma concentration vs. time curve from time 0 to 24 h; V_z , volume of distribution based on the terminal phase; Cl , observed total plasma clearance; $AUMC_{0-24}$, area under the first moment curve from time 0 to 24 h; MRT_{0-24} , mean residence time from time 0 to 24 h; V_{ss} , volume of distribution at steady state.

Table 2. Pharmacokinetic parameter estimates of glycopyrrolate, determined using a three-compartmental model, following intravenous administration of 1 mg to eight ($n = 8$) healthy adult Thoroughbred horses

Parameter	Horse								Median	Min	Max
	1	2	3	4	5	6	7	8			
A (ng/mL)	9.72	5.96	7.49	27.3	9.24	22.8	4.51	7.32	8.37	4.51	27.3
B (ng/mL)	0.436	0.076	0.371	2.23	0.281	0.954	0.935	0.331	0.404	0.076	2.23
C (ng/mL)	0.012	0.069	0.011	0.014	0.011	0.098	0.015	0.071	0.011	0.069	0.015
Alpha (h^{-1})	9.16	6.77	7.30	17.3	8.73	23.5	10.8	8.86	9.01	6.77	23.5
Beta (h^{-1})	1.73	0.809	1.53	3.86	1.57	2.95	2.45	1.09	1.65	0.809	3.86
Gamma (h^{-1})	0.101	0.063	0.095	0.119	0.092	0.102	0.080	0.056	0.094	0.056	0.119
C_{max} (ng/mL)	10.2	6.04	7.88	29.5	9.54	23.8	5.46	7.66	8.71	5.46	29.5
V_1 (L/kg)	0.201	0.302	0.223	0.065	0.179	0.073	0.336	0.239	0.212	0.065	0.336
K_{21} (h^{-1})	2.05	0.884	1.80	4.88	1.79	3.78	3.89	1.43	1.93	0.884	4.88
K_{31} (h^{-1})	0.110	0.070	0.104	0.125	0.101	0.110	0.098	0.062	0.102	0.062	0.125
K_{10} (h^{-1})	7.08	5.58	5.69	13.0	7.03	17.1	5.58	6.10	6.56	5.58	17.1
K_{12} (h^{-1})	1.12	0.505	0.838	2.60	0.830	4.32	2.58	1.76	1.44	0.505	4.32
K_{13} (h^{-1})	0.628	0.603	0.494	0.668	0.646	1.23	1.19	0.656	0.651	0.494	1.23
K_{10-HL} (h)	0.098	0.124	0.122	0.053	0.099	0.041	0.124	0.114	0.106	0.041	0.124
$t_{1/2\alpha}$ (h)	0.076	0.102	0.095	0.040	0.079	0.030	0.064	0.078	0.077	0.030	0.102
$t_{1/2\beta}$ (h)	0.401	0.857	0.454	0.180	0.441	0.235	0.283	0.635	0.421	0.180	0.857
$t_{1/2\gamma}$ (h)	6.89	11.0	7.28	5.82	7.52	6.77	8.61	12.5	7.40	5.82	12.5
AUC ₀₋₂₄ ($h \cdot ng/mL$)	1.43	1.08	1.39	2.27	1.36	1.39	0.979	1.26	1.37	0.979	2.27
Cl_t (mL/min/kg)	23.8	28.0	21.1	14.2	20.9	20.7	31.2	24.3	22.4	14.2	31.2
AUMC ₀₋₂₄ ($h^2 \cdot ng/mL$)	1.47	1.98	1.52	1.20	1.52	1.09	2.43	2.65	1.51	1.09	2.65
V_{ss} (L/kg)	1.46	3.07	1.39	0.449	1.41	0.967	4.64	3.08	1.43	0.449	4.64
V_2 (L/kg)	0.110	0.172	0.104	0.035	0.083	0.083	0.222	0.295	0.107	0.035	0.295
V_3 (L/kg)	1.15	2.60	1.06	0.349	1.14	0.812	4.08	2.54	1.15	0.349	4.08

A, B, and C, intercepts at $t = 0$ for the model equation; alpha, beta, and gamma, slopes for the model equation; C_{max} , extrapolated plasma glycopyrrolate concentration at time 0; V_1 , V_2 , V_3 , volumes of the central, second and third compartments, respectively; k_{21} , k_{31} , k_{12} , k_{13} , distribution rate constants; k_{10} , elimination rate constant; $t_{1/2\alpha}$, distribution half-life; $t_{1/2\beta}$, rapid elimination half-life; $t_{1/2\gamma}$, slow elimination half-life; AUC, area under the plasma concentration vs. time curve; Cl_t , total plasma clearance; AUMC, area under the first moment curve; V_{ss} , volume of distribution at steady state.

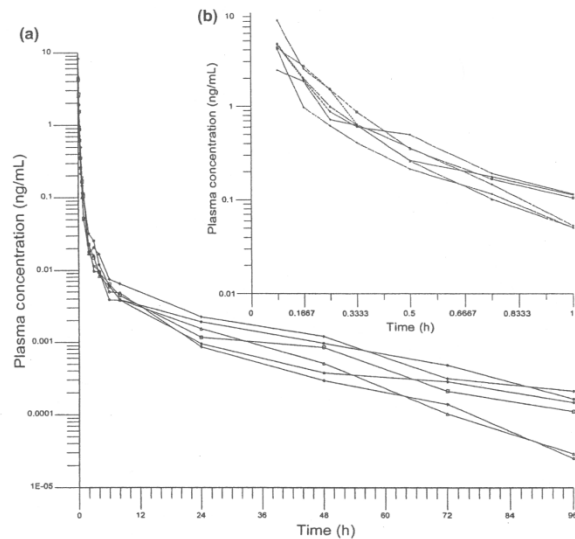


Fig. 1. Plasma concentration (ng/mL) vs. time (h) data from (a) 0–96 h and (b) 0–1 h for glycopyrrolate administered intravenously in eight healthy adult Thoroughbreds.

estimate renal clearance of glycopyrrolate. The total plasma clearance of glycopyrrolate from this study is approximately equal to previous estimates of hepatic blood flow in the horse (Dyke *et al.*, 1998), suggesting that renal clearance may be relatively small. Furthermore, studies in humans, following a single intravenous dose, estimate plasma clearance values to be 16.8 ± 3.83 (mean \pm SD) and 18.1 (10–23.8) (median and range) mL/min/kg (Rautakorpi *et al.*, 1998 and Pentilla *et al.*, 2001), closely approximating human hepatic blood flow (Davies *et al.*, 1993).

In conclusion, plasma pharmacokinetics of glycopyrrolate in the horse following a single intravenous clinically relevant dose can be characterized by a three-compartment model. A wide distribution from the central compartment, rapid clearance, and prolonged terminal half-life were observed. Further studies are needed to determine the extent of the contribution of renal clearance and plasma hydrolysis to the total plasma clearance. We believe the current study contributes reliable data upon which to recommend a withdrawal time and threshold limit for the therapeutic use of glycopyrrolate in racing horses.

ACKNOWLEDGMENTS

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REFERENCES

- Beaufort, T., Proost, J., Kuizenga, K., Houwertjes, M., Kleef, U. & Wierda, J. (1999) Do plasma concentrations obtained from early arterial blood sampling improve pharmacokinetic/pharmacodynamic modeling? *Journal of Pharmacokinetics and Biopharmaceutics*, **27**, 173–190.
- Davies, B. & Morris, T. (1993) Physiological parameters in laboratory animals and humans. *Pharmaceutical Research*, **10**, 1093–1095.
- Dyke, T., Hubbell, J., Sams, R. & Hinchcliff, K. (1998) Hepatic blood flow in horses during the recuperative period from maximal exercise. *American Journal of Veterinary Research*, **11**, 1476–1480.
- Gabrielsson, J. & Weiner, D. (2007) *Pharmacokinetic and Pharmacodynamic Data Analysis, Concepts and Applications*. 4th edn. Sweden: Swedish Pharmaceutical Press.
- Gibaldi, M. & Perrier, D. (1982) *Pharmacokinetics*, 2nd edn. New York: Marcel Dekker.
- Pentilla, J., Helminen, A., Luomala, K. & Scheinin, H. (2001) Pharmacokinetic–pharmacodynamic model for the anticholinergic effect of glycopyrrolate. *European Journal of Clinical Pharmacology*, **57**, 153–158.
- Rautakorpi, P., Manner, T., Ali-Melkkila, T., Kaila, T., Olkkola, K. & Kanto, J. (1998) Pharmacokinetics and oral bioavailability of glycopyrrolate in children. *Pharmacology and Toxicology*, **83**, 132–134.
- Rumpler, M., Sams, R. & Colahan, P. (2010a) Method development and validation of glycopyrrolate in horse urine. Proceedings of the International Conference of Racing Analysts and Veterinarians (ICRAV), Queenstown, New Zealand.
- Rumpler, M., Sams, R. & Colahan, P. (2010b) Validation of an LC/MS/MS method for quantification of glycopyrrolate in horse plasma. *Journal of Analytical Toxicology* (in press).
- Toutain, P. & Bousquet-Melou, A. (2004) Volumes of distribution. *Journal of Veterinary Pharmacology and Therapeutics*, **27**, 441–453.
- Yamaoka, K., Nakagawa, T. & Uno, T. (1978) Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. *Journal of Pharmacokinetics and Biopharmaceutics*, **6**, 165–175.

Lynn Hovda

From: Dr. Scot Waterman [swaterman@rmtcnet.com]
Sent: Tuesday, November 09, 2010 11:41 AM
To: Lynn.Hovda@state.mn.us
Subject: Re: FW: Thresholds

2.0 picograms/ml in plasma. Urine wasn't calculated but I can probably get it if needed.

On Tue, 9 Nov 2010 11:22:50 -0600

Lynn Hovda <Lynn.Hovda@state.mn.us> wrote:

>
>
> Found the upcoming threshold list.
>
>
> Can you shoot me a 48 hour threshold for glycopyrrolate? I know you
> have
> it.

> Lynn

>
> From: Lynn Hovda [mailto:Lynn.Hovda@pop3.state.mn.us]
> Sent: Tuesday, November 09, 2010 10:11 AM
> To: swaterman@rmtcnet.com
> Subject: Thresholds

> Scot:

> What things about the Kentucky meeting would you have changed?

> And what are the numbers after each medication on your list?

> Finally, what meds are you hoping to release thresholds for in April?

Absence of detectable pharmacological effects after oral administration of isoxsuprine

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Keywords: horse; isoxsuprine; behaviour chamber; sweat production; heart rate; temperature; muscle tone; cutaneous blood flow; pharmacological effect

Summary

Isoxsuprine is reported to be a peripheral vasodilator used in human and veterinary medicine to treat ischaemic vascular disease. In horses, it is generally administered orally to treat navicular disease and other lower limb problems. To define the scope and duration of its pharmacological responses after oral administration, 6 horses were dosed with isoxsuprine HCl (1.2 mg/kg bwt) q. 12 h for 8 days and then tested to assess the duration and extent of pharmacological actions. There was no significant difference between isoxsuprine and control treatment values for heart rate, spontaneous activity, sweat production, anal muscle tone, core and skin temperatures, and cutaneous blood flow. The lack of pharmacological effect following oral administration was in sharp contrast to the marked response following i.v. dosing reported in earlier experiments.

Introduction

Isoxsuprine is a vasodilator drug used for cerebral vascular insufficiency and to control premature labour in man (Menard 1984). In veterinary medicine, it has been recommended for the treatment of navicular disease and other lower limb problems in horses (Turner and Tucker 1989; Wilson and Bolhuis 1996).

In a previous study, when isoxsuprine HCl was administered i.v. significant effects were observed on behavioural and physiological variables, including heart rate, spontaneous activity, sweat production, core and skin temperatures, and anal muscle tone (Harkins *et al.* 1996). These findings were consistent with those of another study measuring the effects of i.v. isoxsuprine which showed transient decreases in systemic blood pressure, vascular resistance, and stroke volume, along with transient increases in heart rate, cardiac output, and purposeful movement (Matthews *et al.* 1986). Although the observed effects were marked, the duration of pharmacological action was short and all measurable effects returned to control values within 4 h of

administration of isoxsuprine.

Despite the relatively short duration of pharmacological action, isoxsuprine is one of the more frequently detected therapeutic agents in racing horses (R. Gown, personal communication) and has been detected 42 days after the last dose has been administered (Kellon and Tobin 1995). In equine medicine, isoxsuprine is commonly administered by the oral route rather than i.v.

Since the efficacy of a medication can be highly dependant on the route of administration, the objectives of this study were to assess the duration and extent of the pharmacological effects of oral isoxsuprine when administered at the dose and by the route currently used in equine therapeutics.

Materials and methods

Horses

Six mature Thoroughbred mares weighing 413–602 kg were used. The animals were maintained on grass hay and feed (12% protein: a 50:50 mixture of oats and an alfalfa-based protein pellet). They were vaccinated annually for tetanus and given ivermectin quarterly. A routine clinical examination was performed prior to the beginning of these experiments to assure that the animals were healthy and sound. During experimentation, horses were provided water and hay *ad libitum*. All horses were acclimated to their stalls 24 h prior to experimentation, and each horse was used as its own control.

Drug administration

Isoxsuprine HCl powder¹ 1.2 mg/kg bwt was administered in 0.5 cup of sweet feed (oats, corn and barley mixed with molasses) b.i.d. for 8 days. The horses were fed their usual ration of oats immediately after dosing. On the seventh day of dosing, experiments were conducted to determine the pharmacological effects of isoxsuprine. Heart and respiratory rate, sweat production, anal muscle tone, skin and body core temperatures, and cutaneous blood flow to the skin of the pastern were measured concurrently as described below. These experiments

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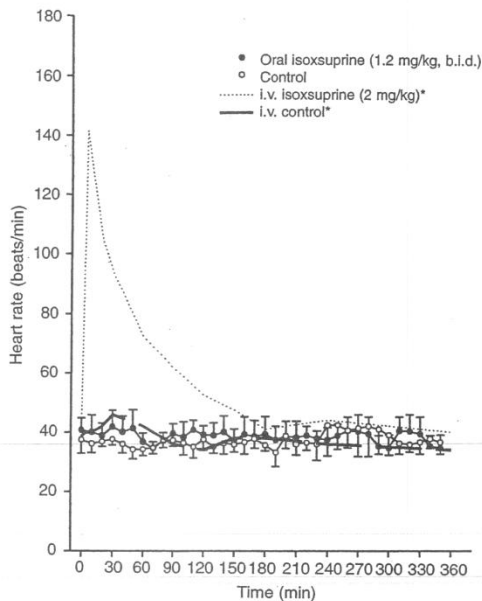


Fig 1: Heart rate following control and oral isoxsuprine treatments. *Harkins *et al.* (1996).

were performed in a specially designed horse stall adapted for physiological measurements. On the eighth day of dosing, the horse was placed in a motion chamber to measure spontaneous movement. The ambient temperature varied between 6.7–21.6°C and relative humidity was 68–100%.

For control values, sweet feed alone was administered. A crossover design would have been preferable; however, the laser Doppler flow meter was designated for teaching purposes, and access to the instrument was limited. However, control flow meter values were obtained 6 weeks later, which should have given sufficient time for any effect of isoxsuprine to have been removed. It was not possible to schedule use of the flow meter more than one or 2 days in advance.

Respiratory and heart rates

Respiratory and heart rates were recorded by clinical observation every 10 min for 6 h after administration of the morning dose on the seventh day of treatment.

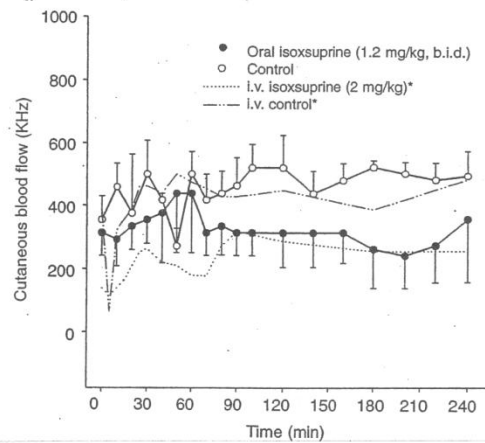


Fig 2: Cutaneous blood flow following control and oral isoxsuprine treatments. *Harkins *et al.* (1996).

Cutaneous blood flow

Cutaneous blood flow was measured at the dorsal pastern with a laser Doppler flowmeter (TSI model 403)². The flowmeter noninvasively measured superficial blood flow (to a depth of 1.2–1.5 mm) by detecting the Doppler frequency shift or laser light reflected by moving red blood cells in the skin. Detailed information on the particular laser Doppler system used has been described previously (Richardson *et al.* 1988). The hair on the pastern was clipped and shaved, the skin was rubbed with isopropyl alcohol, a conducting gel (K-Y Jelly)³ was applied to the skin, and the fibre optic probe was secured with bandaging material. Flow was measured before administration and at 10 min intervals for the first 100 min, then at 20 min intervals until 4 h after administration of the morning dose on the seventh day of treatment.

Sweat production

A plastic sweat catchment device (SCD) was designed to collect all sweat produced following treatments. The SCD extended under the neck anteriorly, fitted securely around both forelegs, extended posteriorly to the front of the stifles, and was suspended about 20 cm below the ventrum of the horse by straps over the withers and lower back. At the lowest point of the SCD, which was located between the front legs, a funnel collected the

TABLE 1: Comparison of mean \pm s.e. peak pharmacological responses after i.v. and oral administration of isoxsuprine HCl. Control i.v. and isoxsuprine i.v. data are from a previous study and are reprinted by permission from Harkins *et al.* (1996)

Variable	Oral control	Oral isoxsuprine	i.v. control	i.v. isoxsuprine
Heart rate (beats/min)	43.1 \pm 2.9	42.3 \pm 4.1	44.3 \pm 3.7	141.6 \pm 5.7*
Cutaneous blood flow (KHz)	520.8 \pm 75.1	437.5 \pm 118.6	500.6 \pm 105.9	315.6 \pm 119.4
Sweat production (ml/5 min)	0.0	0.0	0.0	12.5 \pm 5.6*
Anal muscle tone (psi)	2.2 \pm 0.1	2.9 \pm 0.1	2.3 \pm 0.2	1.2 \pm 0.3*
Skin temperature (°C)	31.5 \pm 0.3	32.1 \pm 0.5	31.1 \pm 0.3	26.7 \pm 0.5*
Core temperature (°C)	37.8 \pm 0.2	37.7 \pm 0.2	37.8 \pm 0.1	37.2 \pm 0.1*
Spontaneous activity (per 5 min)	67.0 \pm 22.9	85.0 \pm 19.6	124.0 \pm 74.5	393.0 \pm 59.0*

*Significant difference ($P < 0.05$) between control and isoxsuprine treatments.

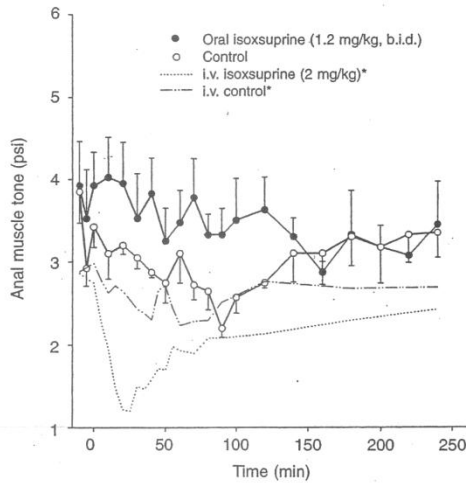


Fig 3: Anal muscle tone following control and oral isoxsuprine treatments. *Harkins *et al.* (1996).

sweat into a graduated cylinder.

Anal tone

A bulb dynamometer⁴ was used to measure changes in anal tone following isoxsuprine treatment. The instrument was originally designed to measure grip strength in arthritic patients, and the size of the bulb (8 cm long, 3.8 cm diameter) was suitable for insertion into the anus to measure maximal muscle contraction. Anal tone was measured before treatment and at 10 min intervals for the first 100 min, then at 20 min intervals until 4 h after administration of the morning dose on the seventh day of treatment.

Skin and core body temperature

Skin temperature was measured with a surface thermistor (Model 409B)⁵ attached to the chest wall with a skin adhesive (Vetbond)⁶. Core body temperature was measured with a general purpose thermistor (Model 401)⁵ placed 50 cm into the rectum and secured to the tail with adhesive tape. Temperatures were monitored by a digital thermometer (Model 8402)⁷ before and at 10 min intervals for the first 100 min, then at 20 min intervals until 4 h after administration of the morning dose on the seventh day of treatment.

Spontaneous activity

The activity of the horse was detected by 4 Mini-beam sensors (SM31E and SM2A31R)⁸ in a specially designed locomotor chamber described previously (Harkins *et al.* 1996). The outputs from the 4 sensors were summed and recorded on a data logger (CR10)⁹ every 5 min. The total number of sensory activations was averaged over a 15 min period. Data were collected for 300 min after administration of the final dose on the eighth day of treatment. Data were expressed as mean step count per 5 min.

Statistical analysis

Data are presented as means \pm s.e. Analysis of variance with

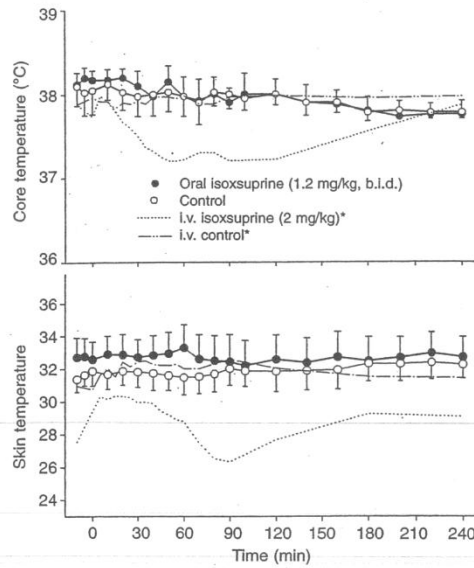


Fig 4: Core and skin temperatures following control and oral isoxsuprine treatments. *Harkins *et al.* (1996).

repeated measures (Anon 1985) was used to compare values for each physiological variable at each measuring time obtained from the control animals with those from the isoxsuprine treated animals. Significance was set at $P < 0.05$.

Results

Following oral administration of isoxsuprine, values for heart and respiratory rates, cutaneous blood flow, anal muscle tone, skin and core body temperatures, spontaneous activity and sweat production were similar to control values and are shown in Figures 1–5. Table 1 contrasts peak values following oral administration of isoxsuprine with corresponding values following i.v. administration (Harkins *et al.* 1996). There were significant differences between oral and i.v. values for all measured variables except cutaneous blood flow.

Discussion

Harkins *et al.* (1996) previously demonstrated that isoxsuprine (2 mg/kg bwt) administered i.v. significantly increased heart rate, locomotor activity, and sweat production. These changes were accompanied by decreased anal smooth muscle tone and decreased skin and core body temperatures consistent with increased heat loss through the skin (Table 1). The observed effects were consistent with an earlier study (Mathews *et al.* 1986) and yielded a coherent picture of the pharmacological actions of isoxsuprine in the horse after i.v. administration. Although the pharmacological effects were marked, they were relatively transient, with all variables returning to control values within 4 h of administration. While these experiments clearly demonstrated the pharmacological effects of isoxsuprine, they did not answer questions concerning the clinical and possible performance-altering effects after oral administration.

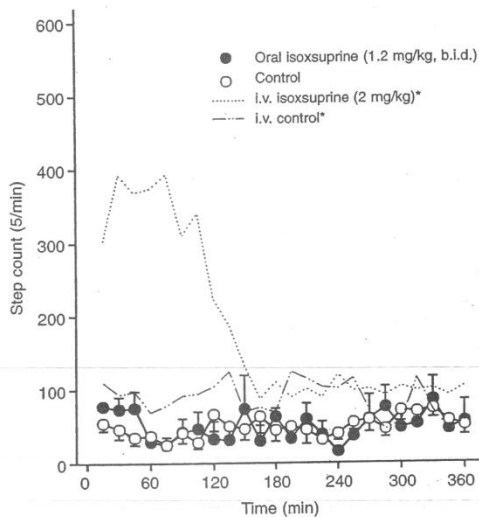


Fig 5: Locomotor activity following control and oral isoxsuprine treatments. *Harkins *et al.* (1996).

Although oral isoxsuprine is well established in clinical practice, its efficacy is poorly supported by objective data. In equine regulatory affairs, the question is even more crucial, since isoxsuprine or its metabolites have been detected in horse urine for up to 6 weeks after administration of the last oral dose, and identifications of isoxsuprine are common in post race urine samples.

The results in this paper show no evidence of pharmacological effects in the horse following 7 days treatment with oral isoxsuprine (1.2 mg/kg bwt b.i.d.; total dose of about 1.2 g/day). This dosage regimen was chosen to reproduce clinical use and the dose was continued for 7 days to ensure that any pharmacological effect was achieved in the test animals. The same evaluation procedures were used as described previously (Harkins *et al.* 1996); however, orally administered isoxsuprine had no effect on heart rate, cutaneous blood flow, sweat production, anal muscle tone, skin and core body temperatures, and spontaneous locomotor activity. The lack of a pharmacological response was markedly different from the responses seen after i.v. administration, when substantial effects were seen on most of the variables measured.

These results are in good agreement with Matthews *et al.* (1986), who reported significant pharmacological and behavioural effects of i.v. isoxsuprine (0.6 mg/kg bwt). In particular, they reported substantial decreases in blood pressure, vascular resistance, and stroke volume, accompanied by increases in heart rate and cardiac output. However, there were no measurable effects after oral administration of this dose b.i.d. for 4 days. Our study, which also showed no pharmacological effects after oral administration of a substantially higher dose for 7 days, both confirms and extends the findings of Matthews *et al.* (1986) concerning the lack of pharmacological effect of oral isoxsuprine. Furthermore, Matthews *et al.* (1986) were unable to detect plasma concentrations of isoxsuprine after oral dosing and concluded that plasma concentrations were insufficient to produce pharmacological responses in the cardiovascular system.

Comparison of the analytical results reported by Matthews *et al.* (1986) with their kinetic data suggests that plasma isoxsuprine concentrations of at least 30 ng/ml are required for pharmacological effects. Although the lower detection limit of their method was not defined, inspection of the data suggests that the peak plasma concentration of isoxsuprine obtained after oral dosing was less than 5 ng/ml.

More recent pharmacokinetic experiments by Joujou-Sisic *et al.* (1996) further support the hypothesis that plasma isoxsuprine concentrations following oral administration are inadequate to produce pharmacological effects. Furthermore, they showed that orally administered isoxsuprine (0.25 mg/kg b.i.d. for 3 days and 0.6 mg/kg b.i.d. on the fourth day) is rapidly conjugated, and that conjugated isoxsuprine was always present in plasma at a higher concentration (up to 500 times greater) than parent isoxsuprine.

A review of the data from Joujou-Sisic *et al.* (1996) suggests that isoxsuprine concentrations of about 50 ng/ml are required for pharmacological effects following i.v. administration. However, orally administered isoxsuprine had an apparent bioavailability of only 2.2%. Peak plasma isoxsuprine concentrations were never higher than 5 ng/ml and declined to less than 1 ng/ml by 1 h after dosing. These findings are consistent with the lack of pharmacological effects of oral isoxsuprine reported in the present study and the earlier experiments of Matthews *et al.* (1986).

The study by Joujou-Sisic *et al.* (1996) also explained how isoxsuprine could be detected for very long periods after its last administration. Since the glucuronide metabolite is cleared very slowly from plasma, isoxsuprine was detected in urine from orally dosed horses for up to 6 weeks after the last administration. This finding is consistent with the well known persistence of residues of isoxsuprine in post race urine samples.

In a recent study, Ingle-Fehr and Baxter (1996) used laser Doppler flowmetry to measure digital and laminar blood flow in normal horses. After the horses were treated orally with 1.2 mg/kg bwt of isoxsuprine, b.i.d. for 10 days, there was no effect on digital and laminar blood flows when measured on Days 2, 4, 7, and 10 of treatment. The authors concluded that the benefits of isoxsuprine treatment for ischaemic conditions of the foot were questionable. Intravenous isoxsuprine (0.6 mg/kg bwt) also failed to increase blood flow in the hoof wall laminae of horses when measured by laser Doppler flowmetry¹ (H.S. Adair, personal communication).

Although the above results show a clear and consistent picture of the pharmacology and pharmacokinetics of isoxsuprine in the horse, they are in apparent conflict with reports of therapeutic efficacy of oral isoxsuprine. Isoxsuprine is used in human medicine and is also used in equine medicine to improve blood flow and circulation in the foot. Clinical studies (Rose *et al.* 1983; Turner and Tucker 1989; Deumer *et al.* 1991; Wilson and Bolhuis 1996) have reported significant therapeutic responses in horses placed on oral isoxsuprine. If oral isoxsuprine is clinically effective and useful in the treatment of navicular disease and similar conditions of the foot, then such effects are subtle, are not identifiable by the methods of pharmacological analysis at our disposal and occur at very low plasma concentrations.

These apparent inconsistencies have been noted by others. Deumer *et al.* (1991) noted that no free isoxsuprine could be detected in the plasma of horses following oral administration, which contrasted with their reported pharmacological activity.

The apparent lack of a pharmacological effect following oral isoxsuprine in horses prompted a careful review of the therapeutic efficacy and pharmacokinetics of oral isoxsuprine in

man. In both man and animals, vasodilation is widely reported following i.v. intra-arterial, and intramuscular administration of isoxsuprine. However, when administered orally, isoxsuprine failed to increase blood flow in the calf, foot, or hand of human subjects. Furthermore, blood pressure was unchanged and there was no difference in heart rate (Zsoter and Baird 1974).

In another study the use of isoxsuprine in man with Raynaud's phenomenon, was assessed using colour thermovision to visualise the degree of circulation in the hands. Following i.v. administration (8 mg), isoxsuprine positively affected various cardiovascular parameters (Wesseling and Wouda 1975). On the other hand, there was no difference in heat emission from the hands and fingers between subjects given control and oral isoxsuprine (20 mg) treatments (Wesseling *et al.* 1981).

Since isoxsuprine stimulates β -adrenergic receptors and causes vasodilation by direct relaxation of vascular smooth muscle, it was used in a study of vasculogenic impotence in men (Knoll *et al.* 1996). After 2 months oral medication (10 mg t.i.d.), the study concluded that oral isoxsuprine was not effective in the treatment of patients with vasculogenic erectile dysfunction.

This present study is the second step in a 3 phase approach to determining the relationship between analytical findings and pharmacological effects of isoxsuprine. The third phase will be the design of kinetic experiments to answer questions concerning the relationship between analytical findings and pharmacological effects (Tobin 1981).

As a practical matter for equine veterinarians, this study and other recent reports raise concerns that clinicians should: 1) rely on their clinical experiences to determine the circumstances for which isoxsuprine appears to be effective, 2) be aware that isoxsuprine metabolites may remain detectable in urine for at least 6 weeks and 3) warn clients about cross-contamination of feed or water to preclude chemical identifications of isoxsuprine in the urine of untreated horses.

In summary, there is limited objective evidence to suggest pharmacological responses to oral isoxsuprine at or below the dose levels used in these experiments in horses. Furthermore, there is compelling pharmacokinetic evidence to suggest that isoxsuprine is subjected to almost complete first-pass metabolism when administered to horses by the oral route. The high extent of first-pass metabolism means that orally administered isoxsuprine is unlikely to attain pharmacologically effective plasma concentrations. In this regard, review of the available pharmacokinetic data suggests that plasma concentrations of at least 30 ng/ml are required for pharmacological effect whereas to date concentrations, reported in the literature, following oral dosing experiments have not risen above 5 ng/ml.

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Manufacturers' addresses

- ¹Sigma Chemical Co, St Louis, Missouri, USA.
- ²St Paul, Minnesota, USA.
- ³Johnson and Johnson Medical Inc, Arlington, Texas, USA.
- ⁴North Coast Medical Inc, San Jose, California, USA.
- ⁵YSI Incorporated Yellow Springs, Ohio, USA.
- ⁶3M Animal Care Products, St Paul, Minnesota, USA.
- ⁷Cole-Parmer Instruments Co, Niles, Illinois, USA.
- ⁸Banner Engineering, Minneapolis, Minnesota, USA.
- ⁹Campbell Scientific, Inc, Logan, Utah, USA

References

- Anon (1985) *SAS Users Guide: Basics*, 5th edn. SAS Institute Inc, Cary, North Carolina. pp 1-1290.
- Deumer, J., deHann, F., Tulp, M.M. and van den Hoven, R. (1991) Effect of isoxsuprine-resin preparation on blood flow in the equine thoracic limb. *Vet. Rec.* **129**, 427-429.
- Harkins, D., Mundy, G.D., Stanley, S., Woods, W.E., Sams, R.A., Richardson, D.R. and Tobin, T. (1996) Character and duration of pharmacologic effects of intravenous isoxsuprine. *Equine vet. J.* **28**, 320-326.
- Ingle-Fehr, J.E. and Baxter, G.M. (1996) The effect of oral isoxsuprine and pentoxifylline on digital and laminar blood flow in healthy horses. *Proc. Am. Ass. equine Practns.* **42**, (Abstract).
- Joujou-Sisic, K., Andren, P.E., Bondesson, U., Fredriksson, E., Larsson, C.I., Kallings, P. and Persson, S.G.B. (1996) A pharmacokinetic study of isoxsuprine in the horse. In: *Proceedings of the International Conference of Racing Analysts and Veterinarians*. Brisbane, Australia. R & W Publications, Newmarket, UK. pp 453-458 (Abstr).
- Kellon, E.M. and Tobin, T. (1995) *Equine Drugs and Vaccines*. Breakthrough Publications, Ossining, New York. pp 99-100.
- Knoll, L.D., Benson, R.C., Bilhartz, D.L., Minich, P.J. and Furlow, W.L. (1996) A randomised crossover study using yohimbine and isoxsuprine versus pentoxifylline in the management of vasculogenic impotence. *J. Urol.* **155**, 144-146.
- Matthews, N.S., Gleed, R.D., Short, C.S. and Burrows, K. (1986) Cardiovascular and pharmacokinetic effects of isoxsuprine in the horse. *Am. J. vet. Res.* **47**, 2130-2133.
- Menard, L. (1984) Tocolytic drugs for use in veterinary medicine. *Can. vet. J.* **25**, 389-393.
- Richardson, D.R., Shephard, S. and McSorley, T. (1988) Evaluation of the role of skin temperature in the response of cutaneous capillary blood flow to indirect heat. *Microcirc. Endothelium Lymphatics* **4**, 447-467.
- Rose, R.J., Allen, J.R., Hodgson, D.R. and Kohnke, J.R. (1983) Studies on isoxsuprine hydrochloride for the treatment of navicular disease. *Equine vet. J.* **15**, 238-243.
- Tobin, T. (1981) *Drugs and the Performance Horse*. Charles C. Thomas, Springfield, Illinois. pp 379-397.
- Turner, A.S. and Tucker, C.M. (1989) The evaluation of isoxsuprine hydrochloride for the treatment of navicular disease: a double blind study. *Equine vet. J.* **21**, 338-341.
- Wesseling, H. and Wouda, A.A. (1975) Effects of intravenously administered isoxsuprine in patients with Raynaud's phenomenon. *Vasa* **4**, 168-174.
- Wesseling, H., denHeeten, A. and Wouda, A.A. (1981) Sublingual and oral isoxsuprine in patients with Raynaud's phenomenon. *Eur. J. clin. Pharmacol.* **20**, 329-333.
- Wilson, J.H.G. and Bolhuis, L. (1996) Controlled clinical studies in Europe with isoxsuprine hydrochloride for the treatment of navicular disease and sesamoiditis in horses. *Ir. vet. J.* **40**, 10-12.
- Zsoter, T.T. and Baird, R.J. (1974) Isoxsuprine as an oral vasodilator. *J. can. med. Ass.* **110**, 1260-1261.

A PHARMACOKINETIC STUDY OF ISOXSUPRINE IN THE HORSE

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ABSTRACT

The β -agonist isoxsuprine was administered iv to Standardbred horses (n=5) as an infusion over 30 min at a dose of 0.6 mg/kg bwt in 500 ml saline. A week later the drug was given orally (n=6) as a powder (Navilox, Univet Ltd, UL) mixed with a small amount of feed. The dosage was 0.25–0.6 mg/kg bwt bid for 4 days. Blood samples were collected just before and at various times to 24 h after the last administration and then every 12 h for a week. Total voided urine was collected for the first 12 h after the iv administration and then daily for a week. During and after the oral treatment urine was collected daily up to 2 weeks. One horse was also sampled after 6 weeks.

Plasma and urine samples were hydrolysed with β -glucuronidase and analysed using a triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionisation (APCI) source. The limit of determination for isoxsuprine was 0.5 ng/ml (c.v. 9%, n=10).

Absorption of the drug appeared to be rapid, taking 15 min to reach the maximum plasma concentration (t_{max}). The mean bioavailability was 2.2 (± 1.5)% and the plasma elimination $t_{1/2}$ was 1.8 (± 0.3) h. Isoxsuprine was distributed outside the vascular compartment. The apparent specific volume of distribution, $V_{d,s}$, was 6.8 (± 1.5) l/kg and the total plasma clearance, Cl, was 2.6 (± 0.5) l/kg/h. On average, 41 (± 13)% was excreted in urine during the first 12 h after iv administration although it was detected for at least 168 h in urine after repeated oral administration. Isoxsuprine was still detectable in one horse after 6 weeks using an enzyme linked immunosorbent assay (ELISA)-technique which was confirmed with liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) at a concentration of 1.5 ng/ml.

INTRODUCTION

Isoxsuprine (4-hydroxy- α -[1-(1-methyl-2-phenoxy-ethyl)amino] ethylbenzenemethanol hydrochloride) is a β -adrenergic receptor agonist that induces peripheral vasodilation (Booth and McDonald 1988). In human medicine, the drug is used to prevent premature labour, hypertension and a variety of ischaemic disorders (Hashem and Lubczyk 1991). In the horse it is used in the treatment of navicular disease, sesamoiditis and occasionally laminitis (Ashelford and Wise 1983; Kirker-Head *et al.* 1986; Deumer *et al.* 1991; Harkins *et al.* 1995). Isoxsuprine has been detected in Standardbred horses following post race urine collection (Ashelford and Wise 1983) and is frequently detected in racehorses in the United States (Harkins *et al.* 1995). In Swedish horseracing, isoxsuprine is a prohibited substance with a withdrawal time of 96 h prior to racing. This withdrawal-period has recently been questioned due to the availability of sensitive analytical methods (Ashelford and Wise 1983; Pompa *et al.* 1994).

The aim of the study was to demonstrate the pharmacokinetic properties of isoxsuprine after a single iv dose and a repeated oral dose.

MATERIALS AND METHODS

Animals

Six Standardbred trotters (geldings) ranging in body weight from 470–550 kg were used. The horses were clinically healthy and were given a diet of hay and oats. They were exercised by running loose in paddocks for 1–2 h daily.

Drug administration

Five horses were administered with isoxsuprine (0.6 mg/kg bwt) iv as an infusion (5 mg/ml in

500 ml saline) over 30 min through a permanent catheter into the jugular vein. The solution was prepared by the Hospital Pharmacy, University Hospital, Uppsala, Sweden.

One week later, the 5 horses and an additional horse were administered isoxsuprine orally as a powder (Navilox, Univet Ltd, UL) mixed with a small amount of feed. The dosage was 0.25 mg/kg bwt bid for 3 days and then 0.6 mg/kg bwt bid on the fourth day.

Sample collection and preparation

Blood samples were collected into heparinised tubes through a permanent catheter placed in the contralateral jugular vein. The first sample was collected just before the iv administration and then at 30, 45, 60, 90 min and 2, 3, 4, 6, 8, 12, 18, 24 h after the start of the infusion. The sampling was continued every 12 h for a week. The horses were then administered isoxsuprine orally and blood samples were collected immediately before the last administration, at 15, 30, 45, 60, 75, 90 min and 2, 3, 4, 6, 8, 12, 18, 24 h after the last administration and then every 12 h for a week. The plasma was separated by centrifugation and stored at -20°C.

Total urine was collected for the first 12 h after the iv administration. Urine samples were collected daily for a week. During and after the oral treatment, urine was collected once a day for 2 weeks and stored at -20°C until analysis. In one horse, urine was collected at 6 weeks.

Analysis

Material: Isoxsuprine was purchased from Sigma (Missouri, USA). Nyliidrin (21 ng/ml) was dissolved in water and used as internal standard for plasma analysis and for urine (1,057 ng/ml). All solvents and reagents were of analytical grade and used without further purification.

Hydrolysis: Plasma or urine (1.0 ml) was added to 1.0 ml of sodium acetate buffer pH 4.8. The mixture was hydrolysed with β -glucuronidase (Helix promatia) overnight at 37°C and 70 μ l of concentrated ammonia solution was added before extraction.

Extraction: The isolation procedure was the same for the unhydrolysed and the hydrolysed samples.

Urine or plasma (1.0 ml) was added to 100 μ l of the internal standard solution. The mixture was adjusted to pH 9.2 by the addition of saturated Na_2CO_3 buffer (1 ml) and the sample was shaken with 6 ml of toluene/2-butanol (8:2 v/v) for 15 min. After centrifugation, the organic phase was

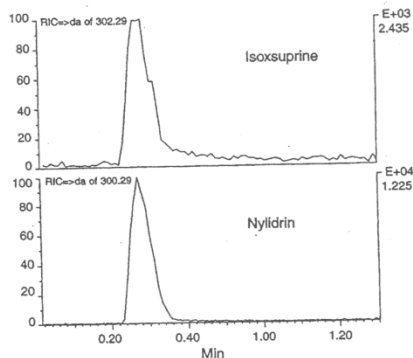


Fig 1: Mass fragmentogram of isoxsuprine, product ion m/z 284 from parent ion m/z 302, and the internal standard nyliidrin, product ion m/z 282 from parent ion m/z 300 (plasma sample).

transferred to another tube containing 1 ml of 0.05 M H_2SO_4 , the mixture was shaken (10 min) and centrifuged. The organic phase was discarded and 1 ml of saturated Na_2CO_3 buffer (pH 9.2) and 5.0 ml of dichloro-methane/2-butanol (8:2) were added. The mixture was shaken for 10 min and, after evaporation, the residue was dissolved in 25 μ l of methanol/acetic acid (99.5:0.5 v/v) before analysis.

Atmospheric pressure chemical ionisation mass spectrometry: A Finnigan TSQ7000 triple quadrupole mass spectrometer (California, USA) equipped with an atmospheric pressure chemical ionisation (APCI) source was used for the analysis of isoxsuprine. The samples were injected through a 5 μ l sample loop attached to an injection valve (Rheodyne model 7725). A Hewlett-Packard model 1050 HPLC was used to pump the mobile phase (methanol - 0.25% acetic acid) and samples by constant infusion at the flow rate of 300 μ l/min.

The mass spectrometer was set in the single reaction monitoring (SRM) mode. The first mass analyser of the tandem mass spectrometer was set to transmit the precursor (parent) ion mass, isoxsuprine m/z 302 and the internal standard, nyliidrin m/z 300, with an acceptance window of about ± 1 m/z units. The precursor ion was dissociated by collision with Ar gas (2.5 mTorr), and the second mass analyser was scanned over a narrow mass range (5 m/z units) for the appropriate product ion (neutral loss of 18 m/z units). Mass measurement accuracy for the product ion was typically ± 0.2 m/z units.

Standard curves: Aqueous standards of isoxsuprine were added at appropriate concentrations to either blank plasma or blank

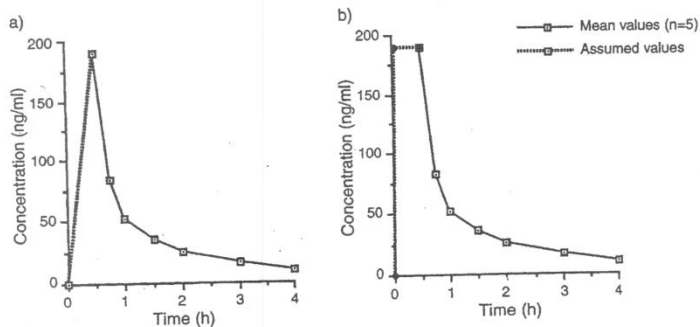


Fig 2. Time concentration profile of isoxsuprine in plasma after iv administration showing the 2 assumed extreme profiles during the infusion; a) the concentration increases to the max value during 30 min; b) the concentration increases almost immediately (3 min) to the highest level and is constant until end of infusion.

urine. These solutions were treated in the same way as the method above. The calibration curves were used to calculate the concentration of isoxsuprine in plasma and urine.

The standard curves of the APCI mass spectrometry/mass spectrometry (MS/MS) system were linear with a correlation coefficient of 0.9995. The limit of determination with the APCI MS/MS was 0.5 ng/ml (cv. 9%, n=10).

The urine and plasma were analysed after extraction prior to analysis by mass spectrometry.

Figure 1 shows a mass fragmentogram of a plasma sample containing isoxsuprine and the internal standard nylidrin. The sample (5 μ l) was eluted in approximately 10 s.

Pharmacokinetics

The plasma concentration versus time data were subjected to non-compartmental analysis based on statistical moment theory (Gibaldi and Perrier 1982). The data were not well described with compartmental modelling because concentration data were lacking as there was no sampling during the infusion time. From the iv study, values were calculated for total body clearance (Cl) and for the apparent specific volume of distribution ($V_{d,\beta}$). The area under the curve (AUC) was determined using the trapezoidal method. The additional area from the last measured concentration to infinity was calculated using the slope of terminal linear phase of the log plasma concentration-time curve.

Because data were missing during the infusion time, a mean value of the AUC was calculated based on 2 extreme situations (Figs 2a and b). The different AUC values did not differ more than 12% from the mean value.

The terminal $t_{1/2}$ was calculated as $0.693/\beta$,

where β is the slope of the terminal phase of the log plasma concentration - time curve. The slope was calculated by linear regression analysis, using the data from an hour and onwards of the log plasma concentration - time curve.

The following equations were used:

$$Cl = \text{dose}/AUC$$

$$V_{d,\beta} = \text{dose}/AUC \times \beta$$

The bioavailability (F) after oral administration was calculated using the values of the areas under the plasma concentration curve for the oral versus the iv isoxsuprine infusion:

$$F = AUC_{\infty PO} / AUC_{\infty IV}$$

RESULTS

Intravenous administration

The time course of isoxsuprine and its glucuronide conjugate concentrations in plasma after iv administration is shown in Figure 3a. The calculated pharmacokinetic parameters are presented in Table 1. The following results are presented as mean values ± 1 sd (n=5). The calculated plasma elimination $t_{1/2}$ of isoxsuprine was 1.8 (± 0.3) h. The drug is extensively distributed outside the vascular compartment because the apparent volume of distribution, $V_{d,\beta}$, was 6.8 (± 1.5) l/kg. The total plasma clearance was approximately 2.6 (± 0.5) l/kg/h.

Side effects including tachycardia, muscle tremor and sweating occurred approximately 10–15 min after the start of infusion and lasted for approximately 20 min after the infusion ceased.

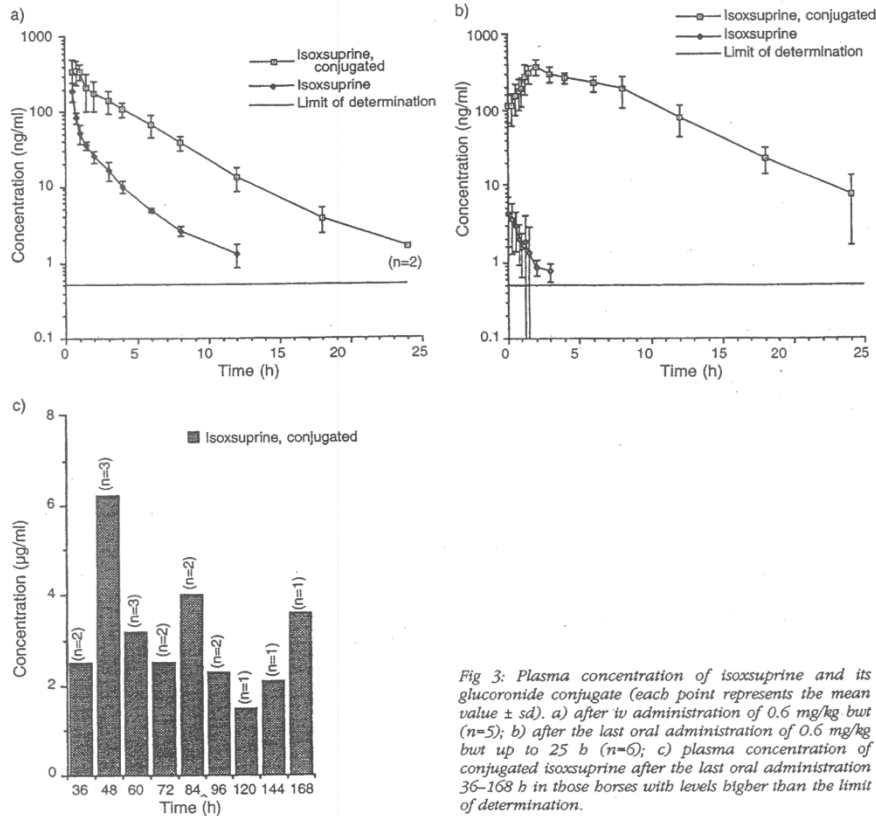


Fig 3: Plasma concentration of isoxsuprine and its glucuronide conjugate (each point represents the mean value \pm sd). a) after iv administration of 0.6 mg/kg but (n=5); b) after the last oral administration of 0.6 mg/kg but up to 25 h (n=6); c) plasma concentration of conjugated isoxsuprine after the last oral administration 36–168 h in those horses with levels bigger than the limit of determination.

Oral administration

The very low mean plasma concentrations of isoxsuprine and its conjugate obtained following the last oral administration are shown in Figure 3b. The following results are presented as mean values \pm sd (n=6). Absorption of isoxsuprine was rapid as the time to reach the maximum plasma concentration (t_{max}) was 15 min. The mean bioavailability (F) was 2.2 (\pm 1.5) %.

The plasma concentration of conjugated isoxsuprine 36–168 h after the last administration is shown in Figure 3c. No adverse reactions occurred after oral administration.

Urine data

The urine concentrations of hydrolysed isoxsuprine after iv and oral treatment are shown in Figure 4.

On average, 41 (\pm 13) % of the drug was excreted in urine during the first 12 h after iv administration although it was detected for a long time in urine after repeated oral administration. A urine sample from a horse 6 weeks after administration was positive with an ELISA-technique. The isoxsuprine (free and conjugated) was identified with LC/MS/MS (1.5 ng/ml).

DISCUSSION

The pathogenesis of navicular disease is unknown but it may be a circulatory disorder. Some studies have attempted to evaluate the effect of isoxsuprine treatment at different doses (Rose *et al.* 1983; Turner and Tucker 1989) with parameters such as skin temperature and gait improvement. There was no improvement when 0.6–1.8 mg/kg bwt isoxsuprine was administered orally (Turner and

TABLE 1: Pharmacokinetic data for isoxsuprine after iv administration

Horse	Clearance (l/h/kg)	V _{d,B} (l/kg)	t _{1/2} (h)
1	2.27	5.06	1.54
2	2.55	8.41	2.29
3	3.42	8.25	1.67
4	2.37	6.15	1.79
5	2.57	6.05	1.63
Harmonic mean	2.58	6.53	1.75
Arithmetic mean (±sd)	2.64 (±0.46)	6.78 (±1.50)	1.78 (±0.30)

Tucker 1989). One effect, an increase in distal limb temperature, was observed 4 h after oral administration which decreased after 8–12 h (Rose *et al.* 1983; Deumer *et al.* 1991). This study showed that the t_{max} is only 15 min and that unconjugated isoxsuprine in plasma was not detectable for more than 8 h after iv administration and 4 h after oral administration.

Isoxsuprine is a β-agonist and *in vitro* studies have shown it to be an α-antagonist with a strong affinity for α-adrenoreceptors which could possibly indicate a potential to affect the distal limb circulation, even at very low plasma levels (Deumer *et al.* 1991). More obvious effects on the heart rate, skin temperature of the front legs and sweating have occurred after iv administration although they return to control values within 4 h (Harkins *et al.* 1995). Similar effects after iv administration were found including an elevated heart rate, muscle tremor and sweating, but the duration was less than 1 h. Pompa *et al.* (1994) detected isoxsuprine in plasma and urine at low levels for a long time, but the pharmacological effects are not evaluated completely (Harkins *et al.* 1995).

The urine and plasma samples were extracted and concentrated in order to clean up the samples and to reduce the concentration of the biological salts, which would interfere with the ionisation process in the APCI source. All analyses were performed by tandem MS (MS/MS) for 2 reasons: in the MS mode, the background ions significantly interfered with measurement of the drug ion, and MS/MS provided much greater specificity for the intact parent compound and lowered the background significantly.

A high degree of conjugation was observed both after iv and oral administration. The low bioavailability could be explained by the high degree of conjugation. Despite the short elimination t_{1/2}, the concentration of isoxsuprine (free and

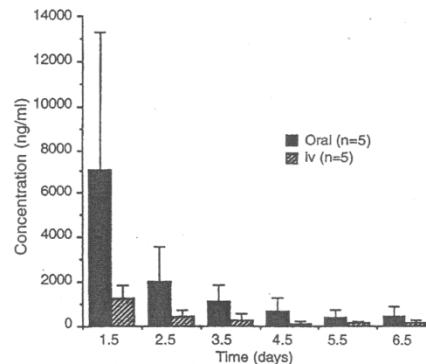


Fig 4: Urine concentrations of hydrolysed isoxsuprine after iv and oral administration (mean values ±sd).

conjugated) was higher in the urine after repeated oral administration than after a single iv administration (Fig 4). After repeated oral administration, the drug could be detected in urine for up to 6 weeks. It is possible that isoxsuprine accumulates in some 'deep body compartment' such as tissue from which it is released slowly. The possible reasons could be strong tissue binding and/or that the tissue is poorly perfused. However, despite the low level of determination of isoxsuprine in plasma, we could not confirm the time course of the redistribution due to the very low plasma concentrations during the redistribution phase.

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REFERENCES

- Ashelford, P.J. and Wise, S.D. (1983) Determination of plasma and urine concentration of isoxsuprine by H.P.L.C. in horses treated with Circulon. *Proc. 5th int. Conf. of use of Drugs in Racehorses*. Ed: G.H. Johnston. Toronto, Canada, pp 179-184.
- Booth, N.H. and McDonald, L.E. (1988) Section 12, *Veterinary Pharmacology and Therapeutics*, 6th edn., Iowa state University Press/ Ames, pp 742-743.
- Deumer, J., de Haan, F., Tulp, M. Th. M. and van den Hoven, R. (1991) Effect of an isoxsuprine-resin preparation on blood flow in the equine thoracic limb. *Vet. Rec.* **129**, 427-429.

-
- Gibaldi, M. and Perrier, D. (1982) *Non Compartmental Analysis Based on Statistical Moment Theory, Pharmacokinetics*. 2nd edn. Ed: J. Swarbrick. Marcel Dekker Inc., New York. pp 409-417.
- Harkins, J.D., Mundy, G.D., Stanley, S., Woods, W.E., Sams, R.A., Richardson, D.R. and Tobin, T. (1995) Character and duration of pharmacological effects of intravenous isoxsuprine. *Proc. Am. Ass. equine Pract.* **41**, 166-167.
- Hashem, A. and Lubczyk, B. (1991) Determination of isoxsuprine in equine plasma by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **563**, 216-223.
- Kirker-Head, C.A., Stephans, K.A., Toal, R.L. and Goble, D.O. (1986) Circulatory and blood gas changes accompanying the development and treatment of induced laminitis. *Equine vet. Sci.* **6**, 293-301.
- Pompa, G., Caloni, F., Montana, M. and Pasqualucci, C. (1994) Prolonged presence of isoxsuprine in equine serum after oral administration. *Xenobiotica*. **24**, 339-346.
- Rose, R.J., Allen, J.R., Hodgson, D.R. and Kohnke, J.R. (1983) Studies on isoxsuprine hydrochloride for the treatment of navicular disease. *Equine vet. J.* **115**, 238-243.
- Turner, S. and Tucker, C.M. (1989) The evaluation of isoxsuprine hydrochloride for the treatment of navicular disease: a double blind study. *Equine vet. J.* **21**, 338-341.

METHOCARBAMOL THRESHOLD STUDY

Richard Sams, PhD
 Director
 HFL Sport Science Laboratory
 Lexington, Kentucky

RMTC Methocarbamol Study

- Introduction
 - Sponsor: Racing Medication and Testing Consortium
 - Title: Pharmacokinetics and Withdrawal Time Estimates for Methocarbamol after Single Dose Intravenous Administration to Thoroughbred Horses
 - Purpose: To determine the pharmacokinetics of methocarbamol and to estimate the withdrawal time of methocarbamol after single intravenous administration of methocarbamol at a clinically relevant dose to athletically conditioned Thoroughbred horses
- Lead investigator: Dr. Richard Sams
- Co-investigator: Dr. Scott D. Stanley
- Preliminary Data: RMTC pilot study conducted in 2007 at UF and UC Davis

RMTC Methocarbamol Study

- Animal Studies
 - Study Center: UF Pharmacokinetics Laboratory in the Equine Performance Laboratory at the University of Florida
 - Study Director: Dr. Patrick Colahan
 - Animal care and use protocol: approved by University of Florida IACUC
 - Facilities: Inspected twice annually by university IACUC team
 - Subjects: Thoroughbred horses housed at UF College of Veterinary Medicine facilities
 - Number of subjects: 20
 - Conditioning: Treadmill exercise three days per week except on dose administration days – able to run one mile in two minutes under conditions required for participation in study
 - Feed and water: Available at all times on study days

Methocarbamol Study

- Withdrawal Study
 - Subjects:
 - Gender: Geldings (11) and mares (9)
 - Weight: 468-605 kg
 - Age: 5-10 years
 - Samples
 - Samples were collected before and at 24, 48, and 72 hours after dose administration
- Pharmacokinetic Study
 - Subjects:
 - Gender: Geldings (6) and mares (0); subset of the subjects in withdrawal study
 - Weight: 505-560 kg
 - Age: 7-10 years
 - Samples
 - Samples were collected before and from 0.08 through 12 hours after dose administration and at 24, 48, and 72 hours after dose administration

RMTC Methocarbamol Study

- Drug Administration Phase
 - Drug: Methocarbamol
 - Source: Wedgewood Pharmacy, compounding pharmacy
 - Formulation: Solution for injection – 100 mg/mL
 - Dose: 15 mg/kg of body weight
 - Route: Intravenous
 - Frequency: Once
 - Sample collections: Individual venipuncture at each collection point into partially-evacuated glass tubes containing LI heparin
 - Sample handling and storage: Plasma was harvested and stored at -70°C until analyzed (less than two months)

RMTC Methocarbamol Study

- Method Development and Validation Phase
 - Method Development Laboratory: Florida Racing Laboratory at the University of Florida
 - Method Development Phase Director: Dr. Richard Sams
 - Analyst: Marc Rumpier
 - Reference: US FDA Guidance Document for Method Validation
 - Method: Liquid-liquid extraction followed by liquid chromatographic-mass spectral analysis of methocarbamol and guaifenesin using stable isotope labeled analogues of the analytes as internal standards.
 - Standards:
 - Methocarbamol: United States Pharmacopoeia and Sigma-Aldrich
 - Methocarbamol- d_2 : Frontier Biopharm
 - Guaifenesin: Sigma-Aldrich
 - Guaifenesin- d_2 , C/D/N Isotopes

*UC Davis meeting
 RMTC Board of Advisors
 October 10, 2011*

RMTC Methocarbamol Study

- Analytical Phase
 - Analytical Laboratory: Florida Racing Laboratory at the University of Florida
 - Analytical Phase Director: Dr. Richard Sams
 - Analyst: Marc Rimpler
- Method: Liquid-liquid extraction followed by liquid chromatographic-mass spectral analysis of methocarbamol and guaifenesin using stable isotope labeled analogues of the analytes as internal standards
- Calibrators: Nine calibrators (0.5, 1, 5, 10, 20, 50, 100, 200, 500 ng/mL) in matrix-matched control plasma in duplicate; prepared fresh on day of analysis. Calibrators contained methocarbamol and guaifenesin.
- Controls: Five positive control samples (1, 25, 75, 250, 400 ng/mL) in duplicate prepared before start of sample analyses and stored under same conditions as study samples. Control samples contained methocarbamol and guaifenesin.
- Samples: Samples were analyzed in duplicate. Samples containing methocarbamol at concentrations greater than 500 ng/mL were diluted with plasma before analysis.

Methocarbamol Study

- Analytical Conditions
 - Column
 - Type: Acquity™ UPLC HSS T3 C18 Analytical Column
 - Dimension: 2.1 mm x 50 mm
 - Particle size: 1.8 μm
 - Temperature: 38 °C
 - Guard Column
 - Type: Acquity™ UPLC HSS T3 Vanguard Pre-column
 - Dimension: 2.1 mm x 5 mm
 - Particle size: 1.8 μm
 - Mobile Phase
 - Mobile phase A: 0.1% formic acid in water
 - Mobile phase B: 0.1% formic acid in methanol
 - Flow rate: 250 μL/min
 - Injection Volume: 20 μL

SRM Acquisition Parameters

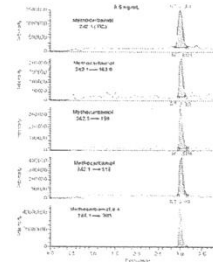
SRM acquisition parameters for methocarbamol and guaifenesin and their deuterated analogues.

Target	Retention Time	Product Ion	Product Ion Ratio	Collision Energy
Methocarbamol	242.1	118	0.20	11
Methocarbamol	242.1	189	0.20	8
Methocarbamol	242.1	163	0.20	13
Methocarbamol-d ₃	248.1	207	0.20	8

Target	Retention Time	Product Ion	Product Ion Ratio	Collision Energy
Guaifenesin	196.0	109	0.20	24
Guaifenesin	196.0	151	0.20	27
Guaifenesin	196.0	167	0.20	11
Guaifenesin-d ₃	202.0	189	0.20	8

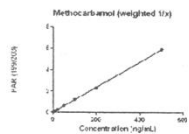
Ion Chromatograms

Total ion chromatogram (upper trace) and ion chromatograms for quantifier ion (m/z 199) and qualifier ions (m/z 163 and 118) for methocarbamol and quantifier ion for methocarbamol-d₃ (m/z 203) (bottom trace). The ion chromatograms were obtained at a methocarbamol plasma concentration of 0.5 ng/mL.



Methocarbamol Determination

Calibration curve for determination of methocarbamol from horse plasma by LC-MS (Thermo Scientific Accela™ liquid chromatograph and Quantum Ultra triple stage quadrupole mass spectrometer with HESI) using methocarbamol-d₃ as internal standard. The calibration curve ranged from 0.5 ng/mL to 500 ng/mL of plasma and was weighted by 1/x where x is the nominal concentration of methocarbamol.



Calibration Data

Table 1. Linear regression analysis data for four sets of calibration data acquired during method validation studies.

Run	Date	Y Intercept	Slope	R ²
Set 1	7/19/2010	0.00299	0.01167	0.9993
Set 2	7/19/2010	0.001368	0.01212	0.9993
Set 3	7/17/2010	0.00266	0.01214	0.9997
Set 4	7/22/2010	0.001343	0.01328	0.9999
Avg		0.002179	0.012748	
St. Dev.		0.00165	0.00097	

Control Samples

Table 2. Individual results for the determination of methocarbamol in control samples analyzed during validation studies are shown below. Four batches of positive control samples were prepared and analyzed over four days. Five replicate samples at each of five concentrations were prepared and analyzed for each batch analysis.

Batch	Replicate	Nominal Concentration				
		1.0 (ng/ml)	2.5 (ng/ml)	7.5 (ng/ml)	250 (ng/ml)	400 (ng/ml)
Batch 1	1	1.03	25.3	77.4	238.9	382.4
	2	1.02	26.1	76.4	246.5	405.7
	3	1.00	24.7	76.1	242.5	410.7
	4	0.98	26.0	75.2	248.3	387.1
	5	1.01	25.2	75.6	241.3	410.8
Batch 2	1	0.98	25.5	76.8	231.8	410.8
	2	0.99	26.2	76.8	241.8	382.0
	3	1.04	27.8	80.9	248.4	408.8
	4	0.98	26.9	79.2	242.8	385.1
	5	1.11	27.3	84.8	236.8	402.8
Batch 3	1	0.98	27.3	77.4	238.9	402.2
	2	1.04	24.0	77.6	235.5	387.3
	3	0.95	27.9	80.6	232.8	405.8
	4	0.98	26.8	79.2	232.3	402.8
	5	1.08	26.5	84.9	238.8	401.4
Batch 4	1	1.07	24.4	73.2	238.5	401.9
	2	0.98	24.3	73.3	238.5	401.1
	3	0.98	23.8	73.3	234.7	407.7
	4	0.92	27.1	78.2	237.7	388.7
	5	1.08	27.7	78.8	232.1	388.8

Intra- and Inter-batch statistics

Table 3. Intra-batch and inter-batch summary statistics for the determination of methocarbamol in control samples analyzed during validation studies are shown below. Four batches of positive control samples were prepared over four days. Five replicate samples at each of five concentrations were prepared and analyzed for each batch analysis.

Batch	Replicate	Nominal Concentration				
		1.0 (ng/ml)	2.5 (ng/ml)	7.5 (ng/ml)	250 (ng/ml)	400 (ng/ml)
Batch 1	1	1.03	25.3	77.4	238.9	382.4
	2	1.02	26.1	76.4	246.5	405.7
	3	1.00	24.7	76.1	242.5	410.7
	4	0.98	26.0	75.2	248.3	387.1
	5	1.01	25.2	75.6	241.3	410.8
Batch 2	1	0.98	25.5	76.8	231.8	410.8
	2	0.99	26.2	76.8	241.8	382.0
	3	1.04	27.8	80.9	248.4	408.8
	4	0.98	26.9	79.2	242.8	385.1
	5	1.11	27.3	84.8	236.8	402.8
Batch 3	1	0.98	27.3	77.4	238.9	402.2
	2	1.04	24.0	77.6	235.5	387.3
	3	0.95	27.9	80.6	232.8	405.8
	4	0.98	26.8	79.2	232.3	402.8
	5	1.08	26.5	84.9	238.8	401.4
Batch 4	1	1.07	24.4	73.2	238.5	401.9
	2	0.98	24.3	73.3	238.5	401.1
	3	0.98	23.8	73.3	234.7	407.7
	4	0.92	27.1	78.2	237.7	388.7
	5	1.08	27.7	78.8	232.1	388.8

Accuracy and Precision

Table 4. Summary statistics for the determination of methocarbamol in control samples analyzed during validation studies are shown below. Five batches of positive control samples were prepared. Four replicate samples at each of five concentrations were prepared and analyzed for each batch analysis. Statistical analysis was performed according to DeChia et al., Pharmaceutical Research 28(11): 1885-1900, 2003.

Characteristic	Batch	Nominal Concentration				
		1.0 (ng/ml)	2.5 (ng/ml)	7.5 (ng/ml)	250 (ng/ml)	400 (ng/ml)
Accuracy	Mean Bias (RMSE)	0.8	3.1	1.8	-0.8	0.2
	UCL	-0.893	0.887	-0.879	-0.805	-0.365
Precision	Inter-batch (CV)	6.0	4.8	1.1	3.8	21.3
	Intra-batch (CV)	6.0	3.2	4.2	3.8	2.1
Accuracy + Precision	(Mean + Inter-batch)	6.833	16.672	6.340	4.623	3.837
95% Expectation	Lower Limit (RMSE)	-0.8	-4.2	4.2	-0.8	-0.3
95% Expectation	Upper Limit (RMSE)	10.8	14.8	10.2	6.1	6.3

Matrix Effects

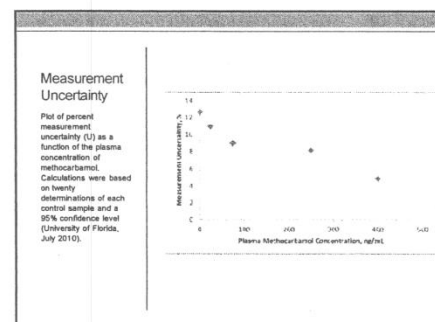
Table 5. Matrix effect, Extraction Efficiency, and Process Efficiency data for methocarbamol in horse plasma determined by the methods described by Matuzewski et al., Anal Chem 75: 3019-3030 (2003).

Batch/Replicate of Concentration	Measured Matrix Effect, %	Extraction Efficiency (%)	Process Efficiency (%)
1	92.00%	78.80%	72.54%
25	104.31%	81.20%	83.90%
75	98.87%	80.10%	80.14%
250	124.30%	83.87%	79.30%
400	124.30%	81.90%	78.60%

Measurement Uncertainty

Table 6. Measurement uncertainty for methocarbamol in control samples by liquid chromatography-mass spectrometry based on data obtained during the validation of the method by Marc Rumples and Richard Sams at the Florida Racing Laboratory, University of Florida (July 2010).

Concentration, ng/ml	n	S _y , ng/ml	RSD, %	k	U, %
1	20	0.06	6.0	2.3	12.4
25	20	1.3	5.18	2.3	10.87%
75	20	3.18	4.25	2.3	8.92%
250	20	9.632	3.86	2.3	8.10%
400	20	9.215	2.3	2.3	4.83



Plasma concentrations from PK study

Plasma methocarbamol concentrations from the six horses pharmacokinetic study as determined by Marc Rumpfer at the UF Racing Laboratory using the validated procedure for determining methocarbamol in horse plasma.

Time (hr)	Horse	Concentration (ng/mL)
0.5	Bob	10.5
0.5	Des	12.1
0.5	Martin	11.8
0.5	Rupert	10.2
0.5	Slip	11.5
0.5	True	10.8
1.0	Bob	8.2
1.0	Des	9.5
1.0	Martin	9.1
1.0	Rupert	8.5
1.0	Slip	9.0
1.0	True	8.8
2.0	Bob	6.1
2.0	Des	7.0
2.0	Martin	6.8
2.0	Rupert	6.2
2.0	Slip	6.5
2.0	True	6.3
4.0	Bob	4.5
4.0	Des	5.2
4.0	Martin	5.0
4.0	Rupert	4.8
4.0	Slip	5.1
4.0	True	4.9
8.0	Bob	3.2
8.0	Des	3.8
8.0	Martin	3.6
8.0	Rupert	3.4
8.0	Slip	3.5
8.0	True	3.3
16.0	Bob	2.1
16.0	Des	2.5
16.0	Martin	2.4
16.0	Rupert	2.2
16.0	Slip	2.3
16.0	True	2.1
32.0	Bob	1.5
32.0	Des	1.8
32.0	Martin	1.7
32.0	Rupert	1.6
32.0	Slip	1.7
32.0	True	1.5
64.0	Bob	1.0
64.0	Des	1.2
64.0	Martin	1.1
64.0	Rupert	1.0
64.0	Slip	1.1
64.0	True	1.0

Sample Analyses

Plot of plasma methocarbamol concentrations in a Thoroughbred horse (Martin) after single dose intravenous administration of 15 mg/kg body weight. Plasma concentrations were determined by a validated LC-MS method characterized by a lower limit of quantification of 0.5 ng/mL. Samples containing methocarbamol at concentrations greater than 500 ng/mL were diluted into the range of the calibration curve (0.5-500 ng/mL). No guanflesin was detected in any post-administration samples.

Pharmacokinetic Analysis

Plasma concentration versus time data were analyzed by non-linear least squares regression analysis using Phoenix[®] WinNonlin[™] software from Pharsight (St. Louis, MO). Data were weighted by 1/C² where y is the measured plasma concentration. A three compartment model provided the best fit of the model to the data for each horse based on AIC and goodness of fit criteria.

Methocarbamol Pharmacokinetic Analysis

Secondary parameters obtained from the pharmacokinetic analysis of plasma concentration versus time data for each horse (n=6). The terminal elimination half-life and mean residence time are relatively short (<4 hours).

Values for the total clearance are about half of hepatic blood flow suggesting that the extent of hepatic metabolism is large and that a high first-pass effect after oral administration should be expected.

Horse	t _{1/2} (hr)	t _{1/2} (min)	CL (mL/min)	MRT (hr)	V _d (L/kg)
Bob	1.04	2.62	12.46	1.44	1079
Des	1.55	3.36	8.60	2.50	1287
Martin	1.48	3.64	8.00	2.45	1178
Rupert	1.05	2.52	11.51	1.83	1265
Slip	1.00	2.45	12.79	1.89	1452
True	1.38	3.06	9.98	2.05	1226

Distribution of plasma methocarbamol

This graph illustrates the distribution of plasma methocarbamol concentrations in samples collected from Thoroughbred research horses 24 hours after intravenous administration of a single dose of 15 mg/kg of body weight to each horse. Plasma concentrations were determined by a validated LC-MS method using a stable isotope labeled internal standard to improve accuracy and precision. The method was characterized by a lower limit of quantification of 0.5 ng/mL. Values ranged from 0.87-11.2 ng/mL.

Transferability

Randomly selected samples and all samples collected at 24 hours were analyzed at the UC Davis laboratory using the method as described in the SOP. Results of the comparison are shown in the figure to the right. The results from UC Davis averaged 22% higher than those reported by UF. However, there is a proportional difference suggesting differences in potencies of standard solutions.

10/17/2011

Methocarbamol Summary

- Plasma methocarbamol concentrations were determined for 24 hours after intravenous administration of a single dose (15 mg/kg of body weight) of methocarbamol to each of twenty Thoroughbred horses.
- Plasma methocarbamol concentrations were determined by a validated UPLC-LCMS method characterized by a method limit of detection of 0.1 ng/mL and a lower limit of quantitation of 0.5 ng/mL.
- The method was validated with regard to accuracy, precision, specificity, sensitivity, linearity, analyte stability, ruggedness, and transferability.
- Plasma methocarbamol concentrations at 24 hours after dosing (n=20) were analyzed and a 99/95 upper tolerance limit of 19 ng/mL was calculated.
- Plasma concentration versus time data in six horses were subjected to PK analysis and a three compartment open model was selected based on AIC and goodness of fit criteria.
- Guafenesin was not detected in any sample collected after intravenous methocarbamol administration.

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12/6/2010

Use of Zilpaterol Earns Most Severe Penalties

Tucson, AZ - The Association of Racing Commissioners International (RCI) today classified Zilpaterol hydrochloride as a "Class 3" substance and called for the most severe penalty should it be found in a racehorse.

The RCI Board called for a minimum penalty of one year suspension and fines in excess of \$10,000 for a first Zilpaterol hydrochloride (Zillmax) violation. A second violation would result in a three year suspension and a minimum fine of \$25,000. A third positive would result in a five year suspension and a \$50,000 fine.

"This drug has no business being in a horse, let alone a racehorse," RCI President Ed Martin said. "There are some who believe they can circumvent the restrictions on steroids by using Zilpaterol. This cannot be tolerated."

Zilpaterol hydrochloride is an adrenergic agonist drug licensed in Mexico and South Africa as a feed additive for cattle at slaughter age. Zilpaterol is a relatively new product and is manufactured by the same company that manufactures ractopamine.

"Neither of these drugs has any business being administered to a horse and certainly the presence of either substance in a horse can only be interpreted as a deliberate attempt to cheat by circumventing the current restrictions on anabolic steroids, as the effect is perceived to be similar," Martin said.

"Based on our review there is no documented medical reason for Zilpaterol hydrochloride to be administered to a horse and therefore if it is found there can be no other motive than a deliberate attempt to violate racing's medication rules," he continued.

The RCI Veterinary Pharmacology Subcommittee, chaired by Dr. Richard Sams, recommended that Zilpaterol be classified as a "Class 3" substances, based upon its pharmacology. The RCI Drug Testing Standards and Practices Committee concurred and the classification was formally adopted today by the RCI Board.

In other action, the RCI Board adopted a recommendation from the Racing

Medication and Testing Consortium to reclassify Dimethyl Sulfoxide (DMSO) as a Class 4 substance with a recommended a plasma threshold of 10 micrograms/ml, which would allow for its use as a topical leg paint but would not allow for the oral or intravenous administration of the drug.

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Analysis of β -agonists by HPLC/ESI-MSⁿ in horse doping control

P. Garcia, A.-C. Paris, J. Gil, M.-A. Popot* and Y. Bonnaire

ABSTRACT: A sensitive method using LC/ESI-MSⁿ has been developed on a quadrupole linear ion trap mass analyser for the detection of nine β_2 agonists (cimaterol, clenbuterol, fenoterol, formoterol, mabuterol, terbutaline, ractopamine, salbutamol and salmeterol) in horse urine. The method consists of solid-phase extraction on CSDAU cartridges before analysis by LC/ESI-MSⁿ. The efficiency of extraction combined with the sensitivity and the selectivity of MSⁿ allowed the detection of these compounds at pg/mL levels. Administration studies of fenoterol and formoterol are reported and show their possible detection after inhalation. The method is applicable for screening and confirmatory analysis. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: β -agonists; HPLC/ESI-MSⁿ; horse urine; SPE extraction

Introduction

Reports on the misuse of β_2 -agonists, both as stimulants and as anabolic agents in sport, emphasize the need for analytical methods for detection of these compounds in doping control. However, most information comes from residue analysis of β_2 -agonists in animal fluids and tissues. β_2 -Agonists act through binding to adrenoreceptors on the smooth muscle cell surface, inducing relaxation of these muscles. β_2 -Agonists are designed for the treatment of pulmonary diseases such as chronic obstructive pulmonary disease, inflammatory airway disease and exercise-induced pulmonary haemorrhage. Clenbuterol (Fig. 1) is a potent selective bronchodilator beneficial for short-term treatment of respiratory disease in the horse. It is the only FDA-approved drug used to prevent horse bronchospasm. At higher doses or after long-term use, β_2 -agonists produce side effects on protein synthesis and lipolysis, resulting in an anabolic effect. Clenbuterol has a history of use as an anabolic agent both in food animals and anecdotally in humans and horses. Other β_2 -agonists like salbutamol and terbutaline (Fig. 1) are currently prescribed for treatment of severe forms of asthma. Owing to their particular structure (Fig. 1), formoterol, salmeterol, fenoterol and mabuterol have longer half-lives. According to Ladaga *et al.* (2000), formoterol (Arterol[™]; marketed in Argentina), has recently been described as an efficient and safe treatment of horse exercise-induced pulmonary haemorrhage.

Cimaterol and ractopamine (Fig. 1) are repartitioning agents (i.e. altering muscle lean to fat ratio). Ractopamine (Paylean[™]) has been approved for use in swine and other livestock as growth regulators. Repartitioning activity results in reduction of fat, increased muscle protein and improved feed efficiency in several species such as swine, cattle and turkeys. Cimaterol and ractopamine are not recognized therapeutic medications for horses but they have a potential for abuse, because they are likely to affect performance. In equine sport all drugs belonging to the class of β_2 -agonists are prohibited. Low doses of these substances are often administered by inhalation, which minimizes side-effects and increases efficiency. Until recently, the detection of β_2 -agonists administered by aerosol was difficult

due to low concentrations of analytes in urine and in plasma. Current screening and confirmation methods are founded on gas chromatography–mass spectrometry. Based on two derivatizations, i.e. trimethylsilyl (TMS) and 2-(dimethyl)silamorpholine (DMS), Dumasia and Houghton (1991) have developed a general method for the screening and confirmation of β -blockers, β_2 -agonists and their metabolites in horse urine. Methyl and *n*-butyl boronate derivatives are also useful to gain sensitivity for detecting certain drugs, such as salbutamol (Zamecnik, 1990). However, this approach is limited to clenbuterol-like compounds and is very efficient for target analysis, such as detection of clenbuterol in urine (Van Eenoo *et al.*, 2002a) or in hair (Popot *et al.*, 2003). A two-step derivatization as cyclic methyl boronate followed by trimethylsilylation was also shown to be powerful for the screening of a larger group of compounds in human urine (Damasceno *et al.*, 2002). Improvements in coupling liquid chromatography with mass spectrometry, in particular by means of atmospheric pressure ionization techniques such as electrospray ionization (ESI), have made possible the development of more reliable procedures for doping analysis, suitable for screening and confirmation in biological matrices.

Regarding sample preparation, as previously described by Poletini (1996), liquid–liquid extraction conducted after hydrolysis is still useful for the extraction of these drugs from plasma and urine (Guan *et al.*, 2002; Van Eenoo *et al.*, 2002a; Di Corcia *et al.*, 2010). Solid-phase extraction (SPE) with mixed mode sorbents is currently employed for the screening of β_2 -agonists in human and animal fluids and tissues, and is still one of the first choices for extraction. Immunoaffinity chromatography has been

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Abbreviations used: CID, collision-induced dissociation; DMS, 2-(dimethyl)silamorpholine; TMS, trimethylsilyl.

proposed for the clean-up of β_2 -agonists (Poletti, 1996). More recently, because of their high specificity, molecular imprinted polymer (MIP) technology has been used successfully for the detection of β_2 -agonists in biological samples (Van Hoof et al., 2005). However, due to its limited commercial availability and high cost, SPE with mixed mode cartridges is preferred.

The purpose of this study was to set up a screening method for the detection of nine β_2 -agonists (Fig. 1) based on SPE and HPLC-ESI/MSⁿ on an LTQ mass spectrometer.

Experimental

Chemicals

HPLC-grade water was obtained by Milli-Q filtration (Millipore, Bedford, MA, USA). Drug standards were purchased from Sigma-Aldrich-Fluka (St Quentin Fallavier, France), Mabuterol was kindly provided by Dr Ph. Delahaut (CER, Marloie, Belgium) and fenoterol by LABERCA (Nantes, France). Analytical-grade solvents were obtained from VWR (Fontenay-sous-Bois, France) and Carlo Erba (Milan, Italy). CSDAU cartridges (3 mL, 500 mg) for SPE were obtained from Interschim (Montluçon, France). β -Glucuronidase and protease came respectively from Roche Diagnostics (Meylan, France) and Sigma-Aldrich-Fluka. Stock solutions of standards were prepared by dissolving the selected analytes in methanol and stored at -20°C before use.

Analytical System

Analyses were performed on an LTQ-XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) connected to an Agilent 1200 series binary pump, equipped with a standard autosampler, a vacuum degasser and a column compartment with a thermostatic control (Agilent, Palo Alto, CA,

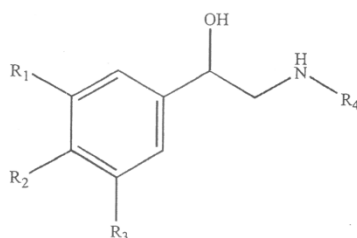


Figure 1. Chemical structures of the target β -agonists. Cimaterol: R₁ = CN; R₂ = NH₂; R₃ = H; R₄ = CH(CH₃)₂. Clenbuterol: R_{1,3} = Cl; R₂ = NH₂; R₄ = C(CH₃)₃. Fenoterol: R_{1,3} = OH; R₂ = H; R₄ = CH(CH₃)-CH₂-C₆H₄OH. Formoterol: R₁ = NH-CHO; R₂ = OH; R₃ = H; R₄ = CH(CH₃)-CH₂-C₆H₄OCH₃. Mabuterol: R₁ = CF₃; R₂ = NH₂; R₃ = Cl; R₄ = C(CH₃)₃. Ractopamine: R_{1,3} = H; R₂ = OH; R₄ = CH(CH₃)-(CH₂)₂-C₆H₄OH. Salbutamol: R₁ = CH₂OH; R₂ = OH; R₃ = H; R₄ = C(CH₃)₃. Salmeterol: R₁ = CH₂OH; R₂ = OH; R₃ = H; R₄ = (CH₂)₆-O-(CH₂)₄-C₆H₅. Terbutaline: R_{1,3} = OH; R₂ = H; R₄ = C(CH₃)₃.

	R ₁	R ₂	R ₃	R ₄
Cimaterol	CN	NH ₂	H	CH(CH ₃) ₂
Clenbuterol	Cl	NH ₂	Cl	C(CH ₃) ₃
Fenoterol	OH	H	OH	CH(CH ₃)-CH ₂ -C ₆ H ₄ OH
Formoterol	NH-CHO	OH	H	CH(CH ₃)-CH ₂ -C ₆ H ₄ OCH ₃
Mabuterol	CF ₃	NH ₂	Cl	C(CH ₃) ₃
Ractopamine	H	OH	H	CH(CH ₃)-(CH ₂) ₂ -C ₆ H ₄ OH
Salbutamol	CH ₂ OH	OH	H	C(CH ₃) ₃
Salmeterol	CH ₂ OH	OH	H	(CH ₂) ₆ -O-(CH ₂) ₄ -C ₆ H ₅
Terbutaline	OH	H	OH	C(CH ₃) ₃

USA). The column used was a Zorbax Eclipse XDB-C₁₈ Solvent Saver Plus (3.5 μm , 150 \times 3.0 mm, 80 \AA), fitted with a Zorbax Eclipse XDB-C₁₈ (5 μm , 12.5 \times 4.6 mm) guard column (Agilent Technologies, Massy, France). The mobile phase consisted of 0.1% formic acid in water (A solvent) and methanol (B solvent). The composition was 90:10 (A : B) from 0 to 1 min, 20:80 (A : B) from 5 to 10 min and 0:100 (A : B) from 11 to 13 min, with column re-equilibration from 14 to 18 min at 100% B. The flow rate was set to 400 $\mu\text{L}/\text{min}$. Aliquots of 5 μL sample were injected. The LTQ was operated in the ESI positive mode (+4.5 kV). ESI source and CID parameters were optimized with all the standard drugs studied at a concentration of 10 ng/ μL in methanol, infused into the analyser at a flow rate of 3 $\mu\text{L}/\text{min}$. The flows of sheath and auxiliary gases were set at 60 and 5 arbitrary units, respectively, in LC conditions. The capillary temperature was maintained at 225 $^\circ\text{C}$. The q_z factor was set to 0.25 for all MSⁿ experiments. The product ions resulting from collision-induced dissociation (CID) of [M + H]⁺ pseudomolecular ions were monitored in the MS³ or MS⁴ mode as described in Table 1. The instrument method for the mass spectrometer is described in Table 2. Each analyte was identified on the basis of at least two product ions obtained in the MS⁵ spectra, according to Table 1.

Drug Administration

Drug administration protocols were established in agreement with animal welfare rules and performed at the administration and sampling centre of Federation Nationale des Courses Francaises (FNCF). After collection, samples were stored at -20°C until analysis. Drug administration by inhalation took place at 9 a.m., 2 h after the morning meal. The device selected for inhalation was a mask Nebul 81 from IPA, Tarare, France.

Fenoterol

A dose of 3.5 $\mu\text{g}/\text{kg}$ of fenoterol, which is in the middle range of therapeutic dosage for a horse, was selected. As a veterinary preparation was not available, a solution of fenoterol in 10 mL of physiological saline was made. Fenoterol was inhaled once by one horse (namely experiment 593). Urine was collected four times over the first 24 h then once every day up to the fourth day after administration.

Formoterol

According to Ladaga et al. (2000) the selected dose of 0.4 $\mu\text{g}/\text{kg}$ of formoterol is a low therapeutic dose. As a veterinary preparation is not available in France, 240 μg of formoterol were dissolved in 10 mL of physiological saline. This solution was inhaled once by one horse (namely experiment 594). Urine was collected four times during the first 24 h then once every day up to the third day after administration.

Clenbuterol

Ventipulmin[™] sirup (Boehringer Ingelheim, Reims, France) was administered orally to one horse at the dose of 1.4 $\mu\text{g}/\text{kg}/\text{day}$ for 10 days (i.e. 0.7 $\mu\text{g}/\text{kg}$ twice a day). In order to avoid any contamination of the stable by clenbuterol, administration was done outside. Urine samples were collected every 12 h for 17 days.

Sample Analysis

Extraction in urine. Five microlitres of internal standard (IS, timolol) dissolved in methanol at the concentration 2 ng/ μL were added to 5 mL of urine and mixed with 1 mL of phosphate buffer (1 M, pH 5.8). After addition of protease (50 μL) and β -glucuronidase (50 μL) hydrolysis was conducted for 1 h at 56 $^\circ\text{C}$. One millilitre of phosphate buffer was added. Centrifugation was carried out for 30 min at 3000g. SPE was carried out on XL4 system (Gilson International France, Roissy) using CSDAU cartridges. The cartridges were conditioned using 2 mL methanol, 2 mL water and 2 mL phosphate buffer. After sample loading, cartridges were washed with 1 mL acetic acid 1 M, dried, washed with 6 mL methanol and dried again. The nine β_2 -agonists were eluted using twice with a 4 mL of

Table 1. Precursor and diagnostic ions (m/z) selected for the detection of the β_2 -agonists by MSⁿ

Analyte	MW (g/mol)	Precursor ion	Diagnostic ions			Method
Cimaterol	219	220	202	160	—	MS ³
Salbutamol	239	240	166	148	—	MS ⁴
Terbutaline	225	226	152	135	125	MS ³
Fenoterol	303	304	286	152	135	MS ³
Clenbuterol	276	277	203	167	132	MS ⁴
Timolol	316	317	261	—	—	MS ³
Mabuterol	310	311	293	237	—	MS ³
Formoterol	344	345	149	121	—	MS ⁴
Ractopamine	301	302	284	164	121	MS ³
Salmeterol	415	416	398	380	232	MS ³

Table 2. Instrument method for the detection of β_2 -agonists in urine samples

Segments duration	Scan events	Parent mass and mass range (m/z)	Analyte
Segment 1 6.30 min	Scan event 1	220 (135–210)	Cimaterol
	Scan event 2	240 (220–300)	Salbutamol
	Scan event 3	226 (115–160)	Terbutaline
	Scan event 4	304 (95–300)	Fenoterol
Segment 2 1.50 min	Scan event 1	277 (120–210)	Clenbuterol
	Scan event 2	317 (180–270)	Timolol
	Scan event 3	311 (220–300)	Mabuterol
	Scan event 4	345 (115–160)	Formoterol
Segment 3 1.20 min	Scan event 5	302 (115–295)	Ractopamine
	Scan event 1	416 (225–410)	Salmeterol

ethyl acetate–32% ammonia mixture (97:3). The residue was finally dissolved in 50 μ L of methanol–water (10:90) and 5 μ L was injected.

Validation

A protocol for the validation of qualitative and quantitative chromatographic methods was followed. For qualitative purposes, the protocol was based on the determination of selectivity/specificity, LOD, recovery and intra-day precision (Jimenez *et al.*, 2002). For a quantitative approach, the protocol was established by evaluation of inter-day precision with the calibration graphs. Recoveries of the nine drugs were determined by the analysis of six urine samples collected from different horses and spiked at 500 pg/mL of each analyte. Matrix effects were studied by analysing 12 different urine samples spiked at 500 pg/mL of each analyte. Between-day variation was determined using a three-day test. Samples were spiked on day 1 with the exception of fenoterol, for which samples were spiked day by day. For clenbuterol, a complementary validation procedure was carried out for quantitative purposes.

Results and Discussion

The analysis of these nine compounds by LC/ESI-MS² has already been investigated (Thevis *et al.*, 2003; Nielsen *et al.*, 2008) on a triple quadrupole mass spectrometer or a 3D ion trap mass spectrometer (Van Hoof *et al.*, 2005). Therefore, in the present study, taking into account previous findings, the method development consisted of the optimization of the parameters on a LTQ-XL mass spectrometer and the selection of the most appropriate MSⁿ method for identification.

Optimization of the Analytical System

With an LTQ-XL mass spectrometer, in order to optimise the detection and improve its repeatability, scan number and maximum injection time were selected so as to obtain six to seven data acquisitions per peak. Fragmentation patterns upon CID for these β_2 -agonists had been elucidated before by LC-ESI/MS² (Thevis *et al.*, 2003; Nielsen *et al.*, 2008). On an LTQ mass spectrometer, in order to make the best compromise between sensitivity and specificity, the selection of the n value (i.e. the value of the scan power) is an important part of the optimization. As shown in Table 1, indicating the choice of MSⁿ scan mode, our selection of diagnostic ions was in accordance with the previous studies. For terbutaline, with the LTQ mass spectrometer in MS³ scan mode, a significant diagnostic ion was displayed at m/z 135 (Fig. 2). This fragment ion originated from the fragment ion at m/z 152 after an NH₃ loss from the side chain. According to the AORC (Association of Official Racing Chemists) recommendation for confirmatory purposes, three diagnostic ions with abundances equal to or higher than 10%, were needed. Consequently, as shown in Table 1 for cimaterol, even though the MS³ scan mode was selected for sensitivity, MS² scan mode was used for confirmatory purposes, because of the presence of three characteristic fragment ions. This is also the case for salbutamol, as indicated in Table 1. The MS⁴ method was selected for sensitivity, but due to the lack of significant fragment ions to ascertain specificity, it was necessary to select the MS³ method for confirmatory purposes. In that case, three diag-

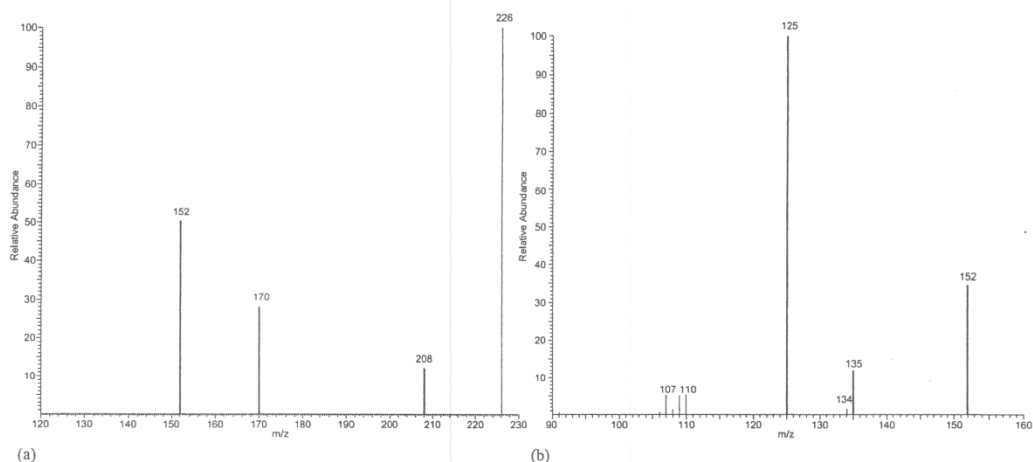


Figure 2. (a) MS² Mass spectrum of terbutaline. (b) MS³ CID mass spectrum of terbutaline. (c) Terbutaline fragmentation pattern showing diagnostic ions.

nostic ions emerged at m/z 148, m/z 166 and m/z 222. The same observation was made for mabuterol, for which the MS² method was preferred rather than the MS³ method. For confirmatory purposes, three diagnostic ions were present at m/z 311, m/z 293 and m/z 237, for which fragmentation pathways of $[M + H]^+$ ion have been described (Guan *et al.*, 2002). In the case of formoterol, the MS³ method had to be selected for confirmatory purposes, the corresponding mass spectrum showing three specific ions at m/z 327, m/z 149 and m/z 121.

Analytical Procedure

The analytical procedure included drug conjugate hydrolysis. These nine drugs were reported mainly as glucuroconjugates (Dumasia and Houghton, 1991; Poletini, 1996; Van Eenoo *et al.*, 2002b; Lehner *et al.*, 2004). Even though liquid–liquid extraction is considered an efficient process, especially for the extraction of clenbuterol (Poletini, 1996; Harkins *et al.*, 2001; Guan *et al.*, 2002), SPE with CSDAU cartridges was preferred for high-throughput screening of β_2 -agonists. According to several authors (Dumasia and Houghton, 1991; Lehner *et al.*, 2001), SPE clean-up was performed using 500-mg Clean Screen, Dau (CSDAU) mixed C₈ and SCX SPE cartridges. In order to optimize recovery of all compounds, the elution step was slightly modified by doubling the elution volume. Regarding the choice of labelled clenbuterol, this did not meet the requirements of a suitable internal standard and therefore timolol was selected.

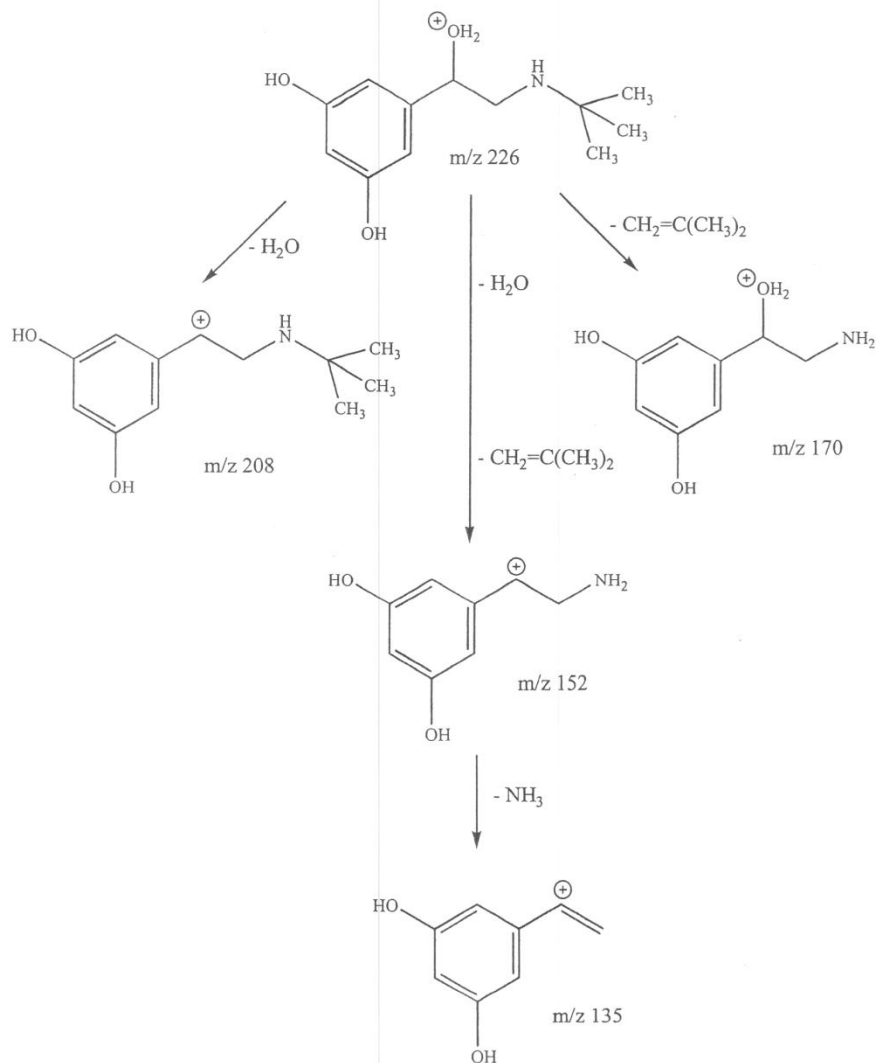
Validation

Retention time differences were below 2% throughout the entire study in accordance with AORC criteria. None of the six blank urine specimens generated interfering signals in the selected ion chromatograms at the expected retention times. This is shown in Fig. 3, which is a superposition of the ion chromatograms obtained from blank urine and those obtained from samples spiked at 100 or 250 pg/mL with fenoterol, formoterol and clenbuterol.

Recoveries varied from 47.7% for formoterol to 97.6% for clenbuterol (Table 3). The variability in matrix factors as measured by the coefficient of variation should be less than 15% for quantitative purposes and 25% for qualitative purposes. Therefore, for qualitative purposes, the data were acceptable for eight drugs out of nine (Table 3). The limit of detection ranged from 9 pg/mL for ractopamine to 80 pg/mL for formoterol, which is at least as low as the limits of detection reported in previous horse studies for salmeterol (Van Eenoo *et al.*, 2002b), clenbuterol (Guan *et al.*, 2002) and ractopamine (Lehner *et al.*, 2004). Calibration curves were prepared by determining the best fit of area ratios (peak area analyte/peak area IS) vs concentration. Back-calculated concentrations were determined and an error percentage equal to or less than 15% was considered acceptable. Precision was determined at the 500 pg/mL level for cimaterol, mabuterol, salbutamol, clenbuterol, formoterol and ractopamine. As shown in Table 4, within-day and between-day precisions were all <15%. For the three other drugs, statistical tests indicated that the selected 500 pg/mL concentration was too low for a quantitative approach. A further precision test was therefore conducted at a concentration of 1.5 ng/mL. It is relevant to notice that data on salmeterol (Van Eenoo *et al.*, 2002b) using liquid–liquid extraction followed by LC/MS analysis were in accordance with our findings. Fenoterol appeared to be unstable in spiked urine samples. Indeed, fenoterol was well detected on the first day, but not detected at all on days 2 and 3. The precision test was therefore repeated by extracting the samples the day of preparation. At a concentration of 1.5 ng/mL, terbutaline, fenoterol and salmeterol passed the test! Intra-day precision was less than 15%. Inter-day precision was less than 15% for terbutaline but higher for fenoterol and salmeterol.

Additional Tests for the Validation of Clenbuterol in Urine

Regarding specificity and selectivity for clenbuterol, retention time differences were below 2% throughout the entire



(c)

Figure 2. Continued.

study. None of the 12 blank urine specimens generated interfering signals at the selected CRM transitions in the MS⁴ scan mode. Detection limit was 25 pg/mL and quantification limit was 75 pg/mL. As reported above, a LOD of 20 pg/mL was found in the previous test. Because this result takes into account a larger baseline, we have considered that the LOD for clenbuterol was 25 pg/mL. A linearity test was conducted, indicating an acceptable fitting from 75 to

1000 pg/mL. At a concentration of 100 pg/mL, accuracy was 108.7%. Within-day and day-to-day precisions were 16.3 and 16.8%, respectively.

Application to Administration Samples

The absence of fenoterol, formoterol and clenbuterol was verified in the urine samples taken before administration.

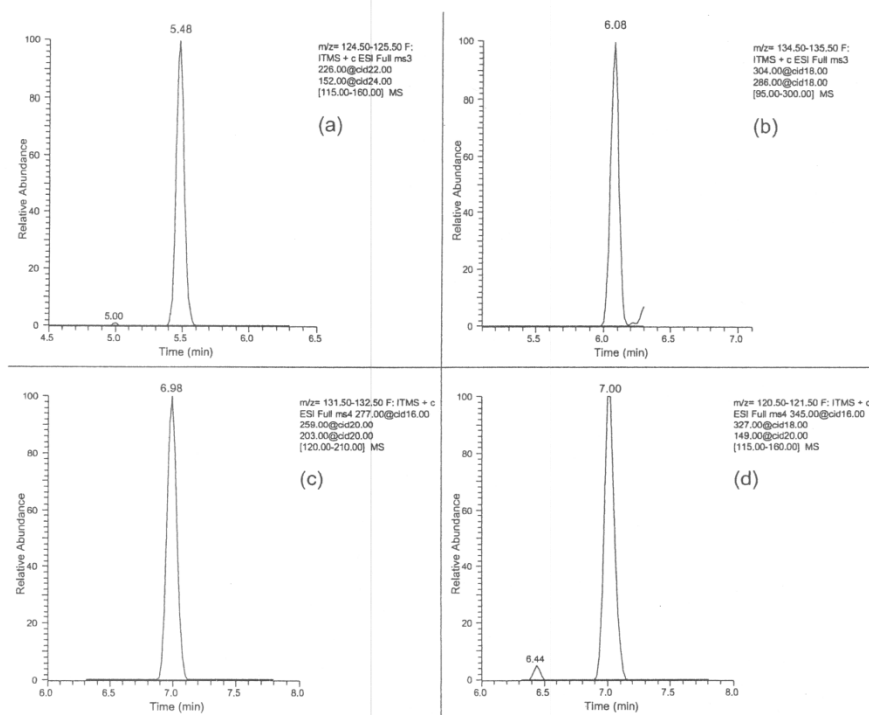


Figure 3. Chromatograms showing the specificity of the method for (a) terbutaline, (b) fenoterol, (c) formoterol and (d) clenbuterol.

Table 3. Recoveries, matrix effect and LOD for the nine β_2 -agonists

Compound	Recovery (% \pm SD)	Matrix effect (%)	LOD (pg/mL)
Cimaterol	73.4 \pm 7.0	6.1	15
Mabuterol	92.6 \pm 5.4	21.5	15
Salbutamol	61.5 \pm 6.9	20.4	20
Terbutaline	66.6 \pm 5.7	23.8	50
Fenoterol	59.9 \pm 8.8	21.6	30
Clenbuterol	97.6 \pm 5.0	14.5	20
Salmeterol	74.4 \pm 14.5	>25	80
Formoterol	47.7 \pm 7.5	16	80
Ractopamine	71.4 \pm 12.0	14	9

Table 4. Precision at 500 pg/mL for six β_2 -agonists

Drug	Intra-day		Inter-day	
	Standard deviation (pg/mL)	Error (%)	Standard deviation (pg/mL)	Error (%)
Cimaterol	28	5.1	39	7.1
Mabuterol	52	7.8	54	8.2
Salbutamol	51	8.7	70	12.0
Clenbuterol	69	11.0	73	11.9
Formoterol	58	10.8	71	13.3
Ractopamine	31	5.3	49	8.4

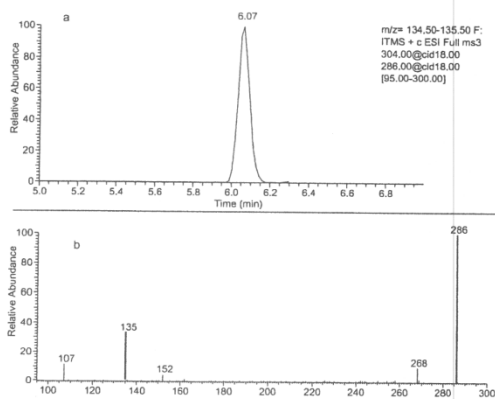


Figure 4. Chromatogram and MS³ CID mass spectrum of fenoterol in a urine sample collected 12 h after its administration by inhalation at the dose of 3.5 $\mu\text{g}/\text{kg}$.

Fenoterol. Later on in the validation process (Table 3), fenoterol was identified in urine samples collected after its administration by nebulisation to one horse (3.5 $\mu\text{g}/\text{kg}$). Chromatogram and MS³ CID mass spectrum of fenoterol (Fig. 4) were obtained from urine collected 12 h after administration. Free and conjugated fenoterol concentrations were estimated in urine collected for 24 h after administration. Peak concentration of approximately 7 ng/mL was observed 3 h after administration. Fenoterol was detectable for about 2 days after administration.

Formoterol. In addition to the validation process (Tables 1 and 2), concentrations of free and conjugated formoterol were estimated in urine. To our knowledge, there are no reports on the detection of formoterol in horse urine after nebulisation. Therefore, the goal of this study was to check if our method was applicable to real samples. The selective ion chromatogram and CID mass spectra obtained from a 24 h post administration sample are suitable to prove the presence of formoterol in urine (Fig. 5a and b). Formoterol was detected for 24 h with a peak concentration of about 5000 pg/mL (Fig. 5c). The method allows the detection of formoterol for at least 24 h. Indeed, in the final urine collection formoterol was detected and estimated at about 200 pg/mL, while LOD was around 80 pg/mL.

Clenbuterol. In this pilot study (Fig. 6), after an oral 10 day treatment at the dose of 1.4 $\mu\text{g}/\text{kg}/\text{day}$, clenbuterol concentration decreased rapidly at the end of the treatment. Urine concentration was less than 1 ng/mL two days after the end of the treatment, but still detectable for more than 10 days post administration. These data are in accordance with previous studies (Guan *et al.*, 2002). The method has been successfully applied for the quantification of clenbuterol in urine collected from 12 other horses. For quantification of clenbuterol in plasma, a slight modification of the SPE elution solvent was made, the LC/MS conditions remaining the same. Owing to the high sensitivity of our method, according to Van Eenoo's findings (Van Eenoo *et al.*, 2002b), application to the detection of inhaled clenbuterol is possible.

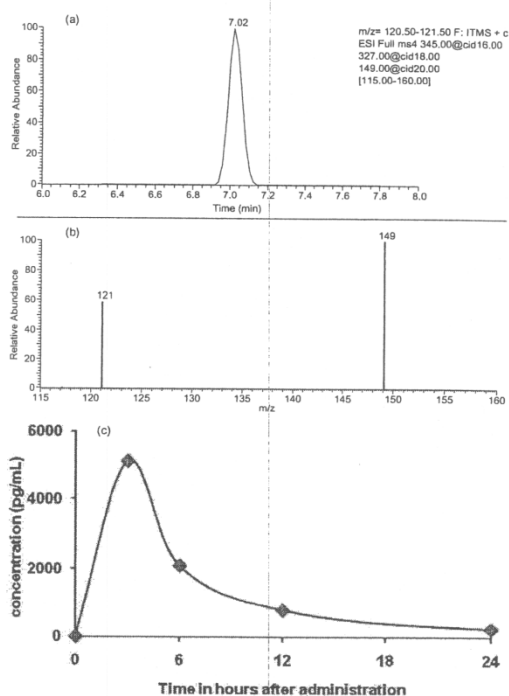


Figure 5. (a) Chromatogram, (b) MS³ CID mass spectrum of formoterol in a urine sample collected 24 h after administration by inhalation at the dose of 0.4 $\mu\text{g}/\text{kg}$. (c) Concentration profile in pg/mL of formoterol in horse urine after its administration by inhalation at the dose of 0.4 $\mu\text{g}/\text{kg}$.

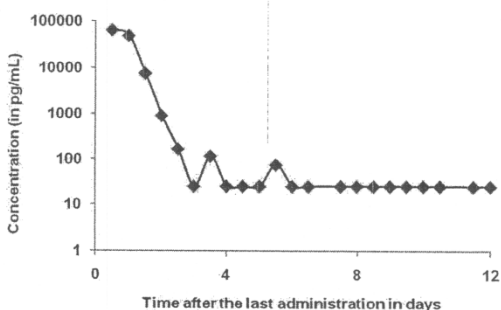


Figure 6. Concentration profile in pg/mL of clenbuterol in horse urine after an oral administration of Ventipulmin[™] syrup at the dose of 0.7 $\mu\text{g}/\text{kg}$ twice a day for 10 days to one horse.

Conclusion

The LC/MSⁿ technique developed on a linear ion trap mass spectrometer has shown that it provides a system useful for the screening and/or the confirmatory analysis of nine β_2 -agonists

in horse urine. The efficiency of extraction combined with the sensitivity and the selectivity of MSⁿ allowed for the detection of these compounds at low levels, which makes possible their detection after inhalation. To our knowledge, this is the first report on an excretion study of fenoterol and formoterol in the horse. Finally, the application of our technique should make it possible to monitor other kinds of drugs such as analgesics like butorphanol and sedatives like fluphenazine and guanabenz.

Acknowledgements

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References

- Damasceno L, Ventura R, Cardoso J and Segura J. Diagnostic evidence for the presence of β -agonists using two consecutive derivatization procedures and gas chromatography-mass spectrometry analysis. *Journal of Chromatography B* 2002; **780**: 61–71.
- Di Corcia D, Morra V, Pazzi M and Vincenti M. Simultaneous determination of β_2 -agonists in urine by fast-gas chromatography mass spectrometry: method validation and clinical application. *Biomedical Chromatography* 2010; **24**: 358–366.
- Dumasia MC and Houghton E. Screening and confirmatory analysis of β -agonists, β -antagonists and their metabolites in horse urine by capillary gas chromatography-mass spectrometry. *J of Chromatography* 1991; **564**: 503–513.
- Guan F, UBoh C E, Soma L R, Luo Yi, Li R, Birks E K, Teleis D, Rudy Ja and Tsang DS. Quantification of clenbuterol in equine plasma, urine, and tissue by liquid-chromatography coupled on-line with quadrupole time of flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 2002; **16**: 1642–1651.
- Harkins JD, Woods WE, Lehner AF, Fisher M and Tobin T. Clenbuterol in the horse: urinary concentrations determined by ELISA and GC/MS after clinical doses. *Journal of Veterinary Pharmacology and Therapeutics* 2001; **24**: 7–14.
- Jimenez C, Ventura R and Segura J. Validation of qualitative chromatographic methods: strategy in antidoping control laboratories. *Journal of Chromatography B* 2002; **767**: 341–351.
- Ladaga GJB, Lebiza FP, Ferraro G and De Erausquin GA. Some pharmacodynamic effects of eformeterol in the horse. *Journal of Veterinary Pharmacology and Therapeutics* 2000; **30**: 496–499.
- Lehner AF, Harkins JD, Karpiesiuk W, Woods WE, Robinson NE, Dirikolu L, Fisher M, Tobin T. Clenbuterol in the horse: confirmation and quantitation of serum clenbuterol by LC-MS-MS after oral and intratracheal administration. *J Anal Toxicol* 2001; **25**: 280–287.
- Lehner AF, Hughes CG, Harkins JD, Nickerson C, Molett B, Dirikolu L, Bosken J, Camargo F, Boyles J, Troppmann A, Karpiesiuk W, Woods WE and Tobin T. Detection and confirmation of ractopamine and its metabolites in horse urine after Paylean administration. *Journal of Analytical Toxicology* 2004; **28**: 226–238.
- Nielen MWF, Lasaroms JJP, Essers ML, Oosterrink JE, Meijer T, Sanders MB, Zuidema T and Stolker AAM. Multi-residue analysis of beta-agonists in bovine and porcine urine feed and hair using liquid chromatography electrospray ionization tandem mass spectrometry. *Analytical Bioanalytical Chemistry* 2008; **309**: 199–210.
- Polettini A. Bioanalysis of β_2 -agonists by hyphenated chromatographic and mass spectrometric techniques. *Journal of Chromatography B* 1996; **687**: 27–42.
- Popot MA, Stojiljkovic N, Garcia P, Bonnaire Y and Tabet JC. First mass spectrometric detection of boldenone in horse mane samples. *Chromatographia* 2003; **57**: 255–260.
- Thevis M, Opferman G and Schänzer W. Liquid chromatography/electrospray ionization tandem mass spectrometric screening and confirmation methods for β_2 -agonists in human or equine urine. *Journal of Mass Spectrometry*, 2003; **38**: 1196–1206.
- Van Eenoo P, Delbeke FT and Deprez P. Detection of inhaled clenbuterol in horse urine by GC/MS². *Biomedical Chromatography* 2002a; **16**: 475–481.
- Van Eenoo P, Deventer K and Delbeke FT. Quantitative detection of salmeterol after inhalation in equine urine by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2002b; **16**: 1755–1759.
- Van Hoof N, Courtheyn D, Antignac JF, Van de Wiele M, Poelmans S, Noppe H and De Brabander H. Multi-residue liquid chromatography/tandem mass spectrometric analysis of beta-agonists in urine using molecular imprinted polymers. *Rapid Communications in Mass Spectrometry* 2005; **19**: 2801–2808.
- Zamecnik J. Use of cyclic boronates for GC/MS screening and quantitation of beta-adrenergic blockers and some bronchodilators. *Journal of Analytical Toxicology* 1990; **14**: 132–136.

ORIGINAL RESEARCH

REFEREED

Adverse Effects of Zilpaterol Administration in Horses: Three Cases

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Carolyn J. Hammer, DVM, PhD,^a Jennifer F. Thorson, BS,^a and David J. Smith, PhD^c

ABSTRACT

Three healthy horses were fed the beta-adrenergic agonist feed additive zilpaterol at a dosage of 0.17 mg/kg body weight to study zilpaterol elimination kinetics. Soon after ingestion of zilpaterol, the horses developed skeletal muscle tremors and tachycardia. A 75 to 87.5% reduced dose of zilpaterol was fed to the horses 24 hours after the initial dose; administration was discontinued thereafter. The horses exhibited restlessness, muscle tremors, and profuse sweating 20 to 25 minutes after ingestion of zilpaterol. Tachycardia developed within 40 minutes and took up to 2 weeks to resolve. Muscle tremors lasted up to 1 week. The most pronounced derangements in serum biochemistry were increased activities of lactic dehydrogenase, creatine kinase, and aspartate transferase, indicating muscle damage. The most severely affected horse also had transient azotemia, hematuria, and proteinuria, suggesting renal damage. All three horses recovered without treatment and were clinically normal 2 to 3 weeks after the initial dose of zilpaterol. Because of their anabolic properties, beta-adrenergic feed additives are considered a risk for abuse in performance horses, despite the absence of Food and Drug Administration approval for such use. Oral administration of zilpaterol to horses at the dosage indicated for use in cattle may result in prolonged adverse effects, including tachycardia, muscle tremors, and renal damage.

Keywords: Horses; Zilpaterol; Adverse effects; Beta-agonists; Tachycardia

INTRODUCTION

Zilpaterol is a beta-adrenergic agonist feed additive approved by the Food and Drug Administration (FDA) in the United States for feeding to cattle to improve weight

gain and enhance carcass leanness. Other beta-adrenergic agonist drugs include ractopamine and clenbuterol. Clenbuterol is the only beta-adrenergic agonist drug approved in the United States for administration to horses, for use in the management of airway obstruction. Because of their muscle-building properties, there is a risk that drugs in this class will be used illegally in performance horses. Consequently, regulatory agencies require data on the disposition of such drugs so that appropriate screening regimens for performance horses may be designed.

To gain more information about the elimination of zilpaterol in the urine of horses, the drug was fed to the three horses described herein. The adverse effects observed, though they were consistent with those expected for beta-adrenergic agonists in horses, were of unexpected severity and duration. Veterinarians and others in the equine industry should be made aware of the risk of prolonged adverse effects when zilpaterol is administered to horses.

CASE REPORT

Three registered Quarter Horses were fed zilpaterol (Zilmax, Intervet Inc, Millsboro, DE) mixed with grain as part of a prospective study designed to evaluate urinary zilpaterol depletion. Horse 1 was a 469-kg, 4-year-old gelding, horse 2 was a 479-kg, 3-year-old filly, and horse 3 was a 462-kg, 5-year-old mare. All three horses had tested negative for the hyperkalemic periodic paralysis gene. The horses were privately owned; the owner had provided informed consent for their use in the study, which was conducted at the Animal Metabolism-Agricultural Chemicals Research Unit (AMACRU) as part of a Native American Internship Program sponsored by the United States Department of Agriculture (USDA-ARS). The study protocol was approved by the Animal Care and Use Committee at the USDA-ARS AMACRU, and the horses were under the supervision of a veterinarian at all times.

After 7 days of acclimation to the research facility and collection of urine and heart rate baseline data, the horses were fed 0.17 mg/kg body weight zilpaterol in 0.5 pounds of grain mix. All horses completely consumed the grain-zilpaterol mixture within 5 minutes of feeding. Within 20 to 25 minutes of being fed the drug, all three horses began to sweat. The horses became agitated and

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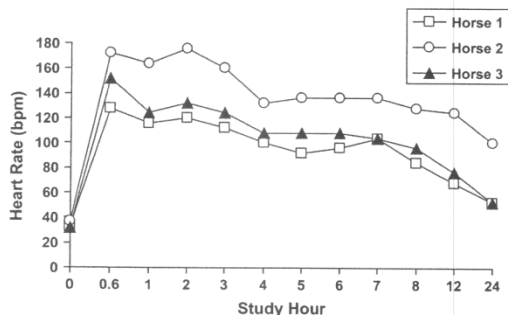


Figure 1. Heart rates in three horses for 24 hours after consumption of 0.17 mg/kg zilpaterol.

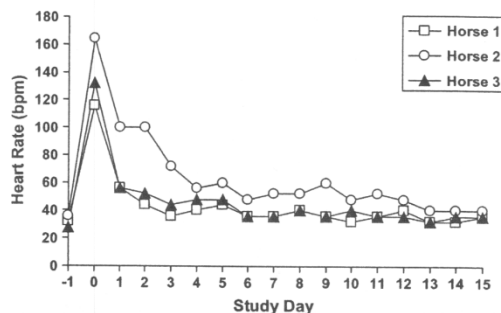


Figure 2. Heart rates in 3 horses for 15 days after consumption of 2 daily doses of zilpaterol.

began to lick and mouth their pens. Horses 1 and 2 became flutulent, and horse 2 exhibited pawing behavior.

All three horses developed markedly increased heart rates within 40 minutes after drug consumption. Heart rates for the three horses during the first 24 hours and for 15 days after initial zilpaterol consumption are shown in Figures 1 and 2. Horse 1, which had a mean baseline heart rate of 33 beats per minute (bpm), had a heart rate of 128 bpm within 40 minutes after feeding, a 387% increase over baseline. Within the same period, heart rates for horse 2 and horse 3 increased from baselines of 37 and 32 bpm to 172 bpm (464% of baseline) and 152 bpm (475% of baseline), respectively. The horses began to sweat profusely, particularly across their necks, shoulders, backs, and flanks. Within 90 minutes of consuming zilpaterol, the horses had developed muscular tremors, which began in the skeletal muscles of the neck, shoulder, and foreleg and spread throughout the visible skeletal muscles. Intermittent visible muscular tremors continued for up to 1 week after the initial dose of zilpaterol.

Twenty-four hours after the initial feeding of zilpaterol, all horses remained tachycardic; horses 1 and 3 had heart rates of 52 bpm, whereas horse 2 had a heart rate of 100 bpm. The adverse effects observed after the first dose were expected to be transient based on the investigators' previous experience with beta-adrenergic agonists in farm animal species and literature reports of the acute effects of other beta-adrenergic agonists in horses. Zilpaterol feeding was therefore continued on the second day of the trial, but at a reduced dosage of 0.043 mg/kg, one fourth the initial dose. Horse 2 did not consume all of the feed that was offered; the dose consumed on the second day by horse 2 was 0.022 mg/kg. Heart rates were not as markedly increased after the second feeding of zilpaterol. Horse 1 peaked at 62 bpm approximately 90 minutes after the second dose, horse 2's heart rate remained at 100 bpm after the second dose, and the heart rate of horse 3 increased

from a pre-feeding level of 52 bpm to a peak of 72 bpm approximately 180 minutes after feeding of the second dose.

One hour after feeding the second dose, blood was drawn from a jugular vein of each horse into a plain glass tube and submitted to the Veterinary Diagnostic Laboratory at North Dakota State University for serum chemistry analysis (VetTest 8008 and VetLyte, IDEXX Laboratories, Westbrook, ME). Horse 1 had mildly elevated activity of aspartate transaminase (AST) and creatine kinase (CK) and mild hypoalbuminemia. Horse 2 had marked elevations in AST, CK, and lactic dehydrogenase (LDH) activity, and glucose, blood urea nitrogen (BUN), and creatinine levels, a mild elevation in alkaline phosphatase (ALP) activity, and mild hyponatremia and hypochloremia. Horse 3 had markedly increased AST, CK, and LDH activity, elevated BUN, creatinine, and glucose, and mild hyponatremia and hypochloremia. Serum chemistry measurements were repeated at intervals over the 2 weeks after initial drug administration; results are summarized in Table 1. Because of the unexpected persistence of adverse effects in these horses, the initial study protocol of 7 consecutive days of drug administration was altered, and drug administration was discontinued after the second dose.

Serum chemistry measurements were repeated in all horses 2, 3, and 8 days after the initial dose of zilpaterol. AST activity decreased over time for all horses, and they all had hypoalbuminemia on days 2, 3 and 8. Horse 2 had a more significantly increased BUN and creatinine than horses 1 and 3, which persisted longer; these values had returned to normal in all horses by 8 days after treatment. Other abnormalities seen in one or more horses on repeat serologic evaluations were elevated ALP and LDH activity, elevated calcium, glucose, and potassium concentrations, and lowered sodium and chloride levels. In horse 1, heart rate returned to baseline levels for the first time 3 days after the initial dose (Fig. 2), and of the 10 observations made on the 4th day, half were at or below baseline

Table 1. Serologic abnormalities observed in three horses after consumption of zilpaterol^a

Days Post-treatment	1 Day			2 days			3 Days			8 Days			14 Days	
	Horse Number	1	2	3	1	2	3	1	2	3	1	2	3	2
Test (Normal Range ^b)														
Albumin (3.8–4.7 mg/dL)	2.8	3.9	3.9	2.9	3.5	2.9	2.9	3	3.1	2.9	2.8	2.8	2.7	2.8
Alkaline phosphatase (10–469 U/L)	170	542	285	153	528	231	140	140	409	256	114	255	206	254
Aspartate transferase (0–317 U/L)	395	657	1,324	384	1,101	1,842	363	>1,083	>1,083	1,886	295	921	1,308	626
Blood urea nitrogen (11–22 mg/dL)	23	37	29	23	57	27	22	60	60	17	21	20	19	20
Calcium (9.9–12.4 mg/dL)	12.5	10.8	12.1	12.5	10.3	11.6	13	10.2	10.9	12.9	12.9	12.9	12.5	12.5
Creatine kinase (1–354 U/L)	503	3,132	^c	171	5,142	1,551	135	4,285	653	116	533	540	211	211
Creatinine (0.4–1.8 mg/dL)	1.7	4.2	2.7	1.5	6.8	1.8	1.6	4	4	1.5	1.5	1.4	1.3	1.5
Gamma-glutamyl transpeptidase (0–50 U/L)	18	39	37	20	46	32	18	39	36	16	16	34	31	32
Globulin (2.4–4.0 g/dL)	4	4	3.7	3.5	4.3	3.8	3.4	3.4	3.7	4.2	3.1	3.5	3.6	3.4
Glucose (58–167 mg/dL)	95	>686	351	93	150	130	91	89	89	98	90	90	84	6.9
Lactate dehydrogenase (0–1337 U/L)	1,162	1,946	2,948	793	>2,800	3,217	830	>2,800	>2,800	2,585	664	2,382	1,803	1,550
Total bilirubin (0.0–2.5 mg/dL)	1.1	1.2	2.3	1	1.8	1.6	1	1	1.7	1.9	0.8	0.8	0.9	0.5
Total protein (5.2–8.5 g/dL)	6.8	7.8	7.6	6.4	7.8	6.7	6.4	6.4	6.8	7.1	5.9	6.3	6.3	6.2
Sodium (132–146 mmol/L)	134	118	123	135	128	127	134	133	133	128	138	142	138	141
Potassium (2.4–4.7 mmol/L)	4.4	2.9	3.1	5.1	2.5	3.7	4.8	<1.5	<1.5	3.4	4.3	4.2	3.6	4.3
Chloride (97–108 mmol/L)	98	78	83	105	85	87	104	86	86	92	104	106	104	106

^a Italicized values are outside normal ranges.^b Reference ranges provided by IDEXX Laboratories, Westbrook, ME for IDEXX VetTest 8008.^c Equipment malfunction: value not available.

level. The heart rate of horse 3 was not more than 12.5% above baseline for half of the observations from day 6 to day 12 after treatment began and returned to baseline on day 13 after the beginning of treatment.

Horse 2, the 3-year-old filly, had the most pronounced and long-lasting clinical signs and serum biochemical abnormalities. Horse 1 did not display a depressed attitude or appetite at any time, and Horse 3 had only a mildly depressed attitude and appetite the day after the first dose. Horse 2, in contrast, had depression and decreased appetite the first day after treatment began, depression on day 2, and decreased appetite on days 2 and 3 after treatment. All observations of heart rate in horse 2 remained at 48 bpm or more, an increase of 30% over baseline, through day 12 after treatment began; heart rate did not drop to baseline level until 16 days after treatment. Because horse 2 had pronounced and prolonged clinical signs and the most serious biochemical derangements, additional diagnostic procedures were performed. Automated complete blood count (CBC) (QBC Autoreader, IDEXX Laboratories, Westbrook, ME) 3 and 4 days after treatment indicated mild leukocytosis and granulocytosis 3 days after the initial treatment and a mild decrease in packed cell volume 4 days after treatment. Eight days after treatment, automated CBC again showed marginally increased platelet count, with rare acanthocytes and neutrophils with toxic changes observed microscopically. Examples of neutrophils with toxic changes to the cytoplasm (basophilia, granularity, and vacuolization), from horse 2, are shown in Figure 3. Automated CBC 14 days after treatment revealed marginally increased white blood cell and platelet counts; on microscopic examination some acanthocytes and neutrophils with toxic changes were observed. Hematologic values for horse 2 are summarized in Table 2. Because all changes were mild, they were considered diagnostically insignificant.

Because of concerns about very high levels of BUN and creatinine in the serum of horse 2, urinalysis was performed on days 2, 3, and 4 post-treatment. On day 2, the urine was dark yellow to brown and had a specific gravity of 1.025, with a pH of 5.0 and high levels of protein, bilirubin, and blood or other heme-like molecules present. On day 3, the urine was yellow and the specific gravity had dropped to 1.016, the pH had increased to 6.0, and high levels of blood or heme-like molecules were still present. By day 4 the urine was light yellow, there were only trace amounts of blood, hemoglobin, or other heme-carrying molecules, and the specific gravity was low at 1.008.

All horses recovered from this adverse drug event without treatment and were clinically normal within 2 to 3 weeks.

DISCUSSION

Zilpaterol is a beta-adrenergic agonist feed additive that was approved by the FDA for use in cattle in the United

States in 2006. Label indications are increased rate of weight gain, improved feed efficiency, and increased carcass leanness in feedlot cattle. In adipose tissue, beta-adrenergic agonists increase blood flow, increase lipolysis, and decrease lipogenesis, whereas in muscle tissue they increase blood flow and protein accretion.¹ Zilpaterol is the second beta-adrenergic repartitioning agent to be approved by the FDA for use in food animals; ractopamine hydrochloride was approved for use in swine in 1999 and for use in cattle in 2003. In the United States, these are the only beta-adrenergic agents approved for use in food animals, and neither drug is approved for use in horses. Another potent beta-adrenergic agonist, clenbuterol, is approved for the management of airway obstruction caused by bronchospasm or mucus accumulation in the airways of horses. The World Anti-Doping Agency considers beta-adrenergic agonist drugs a risk for abuse because of their muscle-building properties and has classified clenbuterol, zilpaterol, and other beta-adrenergic agonists as prohibited for use in human athletes.² There is a risk of abuse in performance animals, too; the beta-adrenergic drug ractopamine has been recovered from the urine of a racehorse in Australia.³

Because of the anticipated abuse of this relatively new beta-adrenergic agonist in performance horses, the original purpose of this study was to measure depletion of zilpaterol residues in urine of horses after a short dietary exposure. The original study protocol described a zilpaterol feeding period of 7 days based on previous work.⁴ There was no intent to measure side effects of zilpaterol because it was expected that side effects would be minor and of short duration, consistent with previously observed effects in horses treated with beta-adrenergic agonists.⁵⁻⁷ In a similar study using sheep dosed with 0.15 mg/kg body weight zilpaterol per day, no side effects were noted, although heart rates were not specifically measured.⁴ The dose of zilpaterol that was chosen for use in the current study is the label dose for cattle of 0.17 mg/kg body weight. Unlike feedlot cattle, which would receive a total dose of 0.17 mg/kg body weight zilpaterol in the ration throughout the day, and the sheep in the previous study, which had the dose divided between two feedings, horses in this study were provided a bolus dose of zilpaterol formulated into a single 227-g grain supplement. Horses were fed the grain before their daily allotment of hay, so the complete zilpaterol dose was given in a single, rapidly consumed portion. The rapid absorption of a bolus dose equivalent to the dose that is usually delivered over a 24-hour period to cattle likely exacerbated the observed adverse effects.

The most commonly reported side effects associated with beta-adrenergic agonist treatment in most animal species are muscle tremors and increased heart rate.^{5,6} In horses, sweating is a side effect that also has been associated with clenbuterol administration.⁷ Symptoms normally decrease

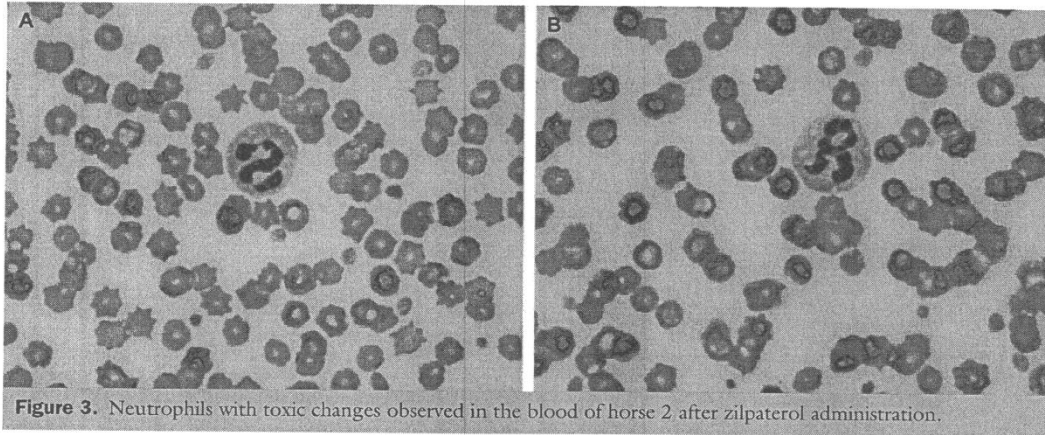


Figure 3. Neutrophils with toxic changes observed in the blood of horse 2 after zilpaterol administration.

Table 2. Complete blood count values for horse 2^a

Test (Normal Range)	Days Post-treatment			
	3 Days	4 Days	8 Days	14 Days
Hematocrit (32.0–52.0%)	40.8	<i>30.9</i>	<i>30.7</i>	<i>32.7</i>
Hemoglobin (11.0–19.0 g/dL)	15.0	11.3	11.3	12.0
White blood cells (6.0–12.5 × 10 ⁹ /L)	<i>14.6</i>	10.4	12.5	<i>13.0</i>
Granulocytes (2.8–8.0 × 10 ⁹ /L)	<i>9.5</i>	5.7	7.5	<i>7.2</i>
Lymphocytes + monocytes (2.1–7.0 × 10 ⁹ /L)	5.1	4.7	5.0	5.8
Platelets (90–350 × 10 ⁹ /L)	337	291	<i>403</i>	<i>356</i>

NOTE. Numbers in italics are outside normal range.

^aReference ranges provided by IDEXX Laboratories, Westbrook, ME, for QBC Autoreader.

over time and resolve within a few hours. In the horses that were fed zilpaterol in this case, the clinical signs observed were unsurprising, but the severity and duration of signs were greater than anticipated. The expectation that clinical signs would be transient, as is typically observed, informed the decision to feed the horses a reduced dose of zilpaterol on the second study day. When it became clear that the adverse effects would not be transient as expected, zilpaterol administration was discontinued.

Increased serum activity of LDH, measured in all three horses, and increased serum activity of CK and AST, measured in the two female horses, are consistent with muscle damage. The increased serum activities of these enzymes in the horses fed zilpaterol, combined with the observation of pronounced, prolonged tachycardia and skeletal muscle tremors, are evidence that some muscle cellular damage resulted from direct or indirect effects of the drug. Moderate to severe muscle damage also causes the release of myoglobin from the muscle tissue, which, like hemoglobin, may bind to the tubules of the kidney, causing tubulonephrosis and related effects such as obstructed

blood flow through the tubules and restricted blood supply to the kidneys. In severe cases, renal failure may result. The brownish color of the urine, marked proteinuria, and marked increases in serum BUN and creatinine in horse 2 could be signs of myoglobinuria and a transient reduction in renal function. Hemoglobinuria appears less likely, because other parameters for hemolysis were not evident. However, the mild decrease in post-treatment packed cell volume (PCV) in combination with color changes of the dipstick indicating bilirubinuria, hemoglobinuria, and proteinuria prevent entirely ruling out the possibility that slight post-treatment hemolysis took place in horse 2. The two female horses also had markedly increased blood glucose the day after they were first fed zilpaterol (>686 mg/dL in horse 2 and 351 mg/dL in horse 3). This finding suggests that these two horses were more sensitive than the gelding to the direct and indirect effects of the beta-adrenergic agonist drug on glucose metabolism. Stress-induced release of catecholamines may have aggravated the hyperglycemia or reduced glucose utilization in peripheral tissues associated with administration of zilpaterol.⁸

Hypoalbuminemia, which was observed in all three horses, is difficult to explain in the presence of normal total protein level; some degree of renal failure appears a plausible cause. Horse 1 was mildly hyperkalemic, which may be because of muscle damage and cell destruction. Horses 2 and 3, which were more severely affected than horse 1, experienced hyponatremia, most likely caused by sodium loss from profuse sweating or impaired renal sodium transport, and hypochloremia, which may be associated with endogenous glucocorticoid release or renal impairment. Horse 2, which was the most severely affected, also had increased serum alkaline phosphatase activity. Whether this was caused by liver impairment, mucosal damage, bone disorder, glucocorticoid release, hyperglycemia, or even by leukocyte dysfunction cannot be answered. All three horses had mildly increased serum levels of calcium; the explanation for this phenomenon is not known.

All of the horses were fed the same initial dose of zilpaterol, whereas the second dose was one-fourth the initial dose in horses 1 and 3 and one-eighth the initial dose in horse 2, because she did not consume all of her feed. Nevertheless, clinical signs and hematologic abnormalities were mildest in the 4-year-old gelding, more pronounced in the 5-year-old mare, which received the same total dosage as the gelding, and most pronounced in the 3-year-old filly, which received a slightly lower total dose than the other two horses. The severity of adverse effects in these three horses did not correlate with the dosage of zilpaterol they consumed and may be related to differences in body composition, sex, and age.

Although direct comparisons of oral potency between zilpaterol and other beta-agonists, such as ractopamine and clenbuterol, cannot be made for horses, human "no observable effect level" data indicate that clenbuterol is approximately 19 times more potent than zilpaterol, and that zilpaterol is about 125 times more potent than ractopamine.⁹ For this reason, it may not be surprising that side effects were not mentioned after bolus administration of either 0.54 or 1.64 mg/kg body weight of ractopamine HCl to horses, doses 3.2 and 9.8 times greater than doses of zilpaterol provided to horses in this study.¹⁰

Pharmacokinetic data that might be helpful in explaining the side effects observed in this study are not available for zilpaterol. Based on zilpaterol's chemical structure and the fixed steric position of its hydroxyl group (rendering it less likely to be a target for glucuronidation or sulfation relative to a phenol), one might predict that zilpaterol would have a serum half-life greater than ractopamine, but less than clenbuterol.¹¹

CONCLUSION

Oral consumption of zilpaterol at the dosage of 0.17 mg/kg body weight produced clinical signs typically associated with beta-adrenergic agonist drug administration, but the duration of the signs and the associated hematologic abnormalities were more pronounced than expected. Because of the potential for extralabel abuse of zilpaterol in performance horses, veterinarians and horse owners should be aware of the possibility that administration of the drug to horses may produce prolonged adverse effects.

ACKNOWLEDGMENTS

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REFERENCES

1. Mersmann JH. Overview of the effects of beta-adrenergic receptor agonists on animal growth including mechanisms of action. *J Anim Sci* 1998;76:160-172.
2. World Anti-Doping Agency. The 2007 Prohibited List, World Anti-Doping Code Available at: www.wada-ama.org/rtecontent/document/2007_List_En.pdf. Accessed August 17, 2007.
3. Racing NSW. 2005 Racing NSW Annual Report Available at: www.racingnsw.com.au/pdf/2005_Annual_Report-screen.pdf. Accessed August 17, 2007.
4. Shelver WL, Smith DJ. Tissue residues and urinary excretion of zilpaterol in sheep treated for 10 days with dietary zilpaterol. *J Ag Food Chem* 2006;54:4155-4161.
5. Spangler DL. Review of the side effects associated with beta agonists. *Ann Allergy* 1989;62:59-62.
6. Nazzal CA. The clinical pharmacology of clenbuterol. *Southwestern Vet* 1985;36:121-125.
7. Boehringer Ingelheim Animal Health, Ventipulmin Syrup Package insert. http://www.bi-vetmedica.com/product_sites/Ventipulmin/documents/ventipulmin_rp.pdf. Accessed August 17, 2007.
8. Skikama H. Adrenergic receptor and epinephrine-induced hyperglycemia and glucose tolerance. *Am J Physiol* 1975;229:962-966.
9. Smith DJ, Turberg MP, Burnett TJ, Dalidowicz J, Thomson TD, Anderson DB. Relative safety of clenbuterol and ractopamine residues in edible tissues of hogs. *Proceedings 17th International Pig Veterinary Society Congress, 2002*;1:194.
10. Lehner AF, Hughes CG, Harkins JD, Nickerson B, Mollett B, Dirikolu L, et al. Detection and confirmation of ractopamine and its metabolites in horse urine after Paylean administration. *J Anal Toxicol* 2004;28:226-237.
11. Smith DJ. The pharmacokinetics, metabolism, and residues of beta-adrenergic agonists in livestock. *J Anim Sci* 1998;76:173-194.

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Exhibit 41

On December 22, 1999, the Food and Drug Administration (FDA) approved ractopamine for finishing swine. Elanco Animal Health, a division of Eli Lilly and Company, has come out with a ractopamine product called Paylean™. There is much "barn talk" about its effects, uses and misuses, and this article will address this issue.

It has been known for years that ractopamine enhances growth in meat animals by repartitioning nutrients. This means that the nutrients that the animal consumes in its feed are shifted away from being stored as fat and are instead used in lean tissue production. This shift occurs as the animal reaches the top of its growth curve and lean tissue production drops off and fat deposition increases. Fat levels also appear to decrease, due to an increase in lypolysis (fat breakdown).

Effects

If you have raised pigs or steers or lambs for very long, you have already figured out that there is no magic pill that will make an average animal into an ideal animal. However, you also know that proper management, nutrition and housing can greatly affect your animals' performance. Paylean™ should only be viewed as a management tool to enhance the genetic potential of your animal. It is not a magic pill and cannot substitute for genetics or proper management.

According to Elanco Animal Health literature, Paylean™ fed at 18 grams per ton improved feed efficiency by 13%, increased average daily gain by 10%, reduced average daily feed intake by 6% and increased lean gain by 25-37% in research trials. However, increased protein, specifically lysine and other amino acids are needed in the diet to enhance lean tissue growth. A diet of at least 16% CP is recommended. Nutritional requirements are greater with the use of ractopamine, but since many show pig diets are excessive in protein and specifically lysine, this is often not a problem. There does not seem to be any effect on muscle quality at the given time. Since show pigs are already extreme in muscling and leanness (therefore possibly closer to reaching their genetic potential), I would not necessarily expect to see such large increases in these production traits, although some improvement is certainly expected.

One of the most important things to consider when using Paylean™ is that *the response is not constant over time*. The largest response will be seen in the first four weeks of feeding, during the repartitioning phase. Therefore, *you do not need to feed it long to get a response*. Feeding it longer than recommended (during the last 7 weeks of the finishing phase) is illegal and will not yield an additive response. It is important to remember that Paylean™ increases growth (both in average daily gain and feed efficiency) and therefore your pigs will grow differently than they would normally. Closely monitoring each individual pig's growth and keeping track of the days to the show will prevent your animal from being over its optimal weight. Carefully plan the use of Paylean™ so that you are within the law, keep the cost to a minimum and maximize results. Abuse of this product could have serious food safety concerns. It is only labeled for swine at this time, *not* steers or lambs.

Label Directions

Paylean™ is approved at 18 grams per ton from 150 to 240 pounds (pen average). This is considered the last seven weeks of the finishing period. There is no withdrawal time when using Paylean™. Upon cessation of using Paylean™, a decrease in performance (back to the performance level prior to using Paylean™) occurs over a period of time (approximately 5-6 weeks). As with any drug, care should be taken to follow the label instructions completely. Increasing the dosage (feeding more than 18 g/T, which is equal to 20 ppm) has shown absolutely no improvement over 18 g/T in either on-farm performance or carcass parameters. Therefore, it appears that a point of diminishing returns is reached when exceeding the dosage level. *Increasing the dosage (above 18 g/T) or feeding longer is not recommended and is, in fact, illegal.*

Questions? Contact Dr. Jodi Sterle at (979) 845-2714 or j-sterle@tamu.edu. A fact sheet is also available from Elanco Animal Health. Their phone number for questions on Paylean™ is 1-800-428-4441.

Net Weight on Bulk Invoice
Ractopamine and Monensin Plus
Type C Medicated Cattle Feed
 For Use in Cattle Only
 Do not feed undiluted.

For increased rate of weight gain, improved feed efficiency, increased carcass leanness, prevention and control of coccidiosis due to *Eimeria bovis* and *E. zuernii* in cattle fed in confinement for slaughter for the last 28 to 42 days on feed.

ACTIVE DRUG INGREDIENTS

Ractopamine HCL.....	9.8 to 24.6 g/ton*
Monensin, USP.....	10 to 40 g/ton*

GUARANTEED ANALYSIS

Crude Protein, not less than.....	_____ %
Non-Protein Nitrogen (NPN) ¹ , not more than.....	_____ %
Crude Fat, not less than.....	_____ %
Crude Fiber, not more than.....	_____ %
Calcium, not less than.....	_____ %
Calcium, not more than.....	_____ %
Phosphorus, not less than.....	_____ %
Salt ² , not less than.....	_____ %
Salt ² , not more than.....	_____ %
Sodium ³ , not less than.....	_____ %
Sodium ³ , not more than.....	_____ %
Potassium, not less than.....	_____ %
Vitamin A ^{2,4} , not less than.....	_____ I.U./lb

¹When added.

²If added.

³Shall be guaranteed only when total sodium exceeds that furnished by the maximum salt guarantee.

⁴Other than precursors of Vitamin A.

INGREDIENTS

Each ingredient must be named in accordance with the names and definitions adopted by the Association of American Feed Control Officials.

FEEDING DIRECTIONS

Feed continuously as sole ration to provide 90 to 430 mg/hd/day ractopamine and 0.14 to 0.42 mg monensin/lb body weight per day, depending on the severity of the coccidiosis challenge, up to 480 mg/hd/day monensin for the last 28 to 42 days on feed.

* Final printed label on formulated Type C medicated feed must bear a single concentration of each drug.

CAUTION

Do not allow horses or other equines access to feeds containing monensin. Ingestion of monensin by horses has been fatal. Monensin medicated cattle feed is safe for use in cattle only. Consumption by unapproved species may result in toxic reactions. Do not exceed the levels of monensin recommended in the feeding directions, as reduced average daily gains may result. If feed refusals containing monensin are fed to other groups of cattle, the concentration of monensin in the refusals and amount of refusals fed should be taken into consideration to prevent monensin overdosing.

Ractopamine HCL is not for animals intended for breeding.

WARNING

A withdrawal time has not been established for pre-ruminating calves. Do not use in calves to be processed for veal.

The active ingredient ractopamine hydrochloride is a beta-adrenergic agonist. Individuals with cardiovascular disease should exercise special caution to avoid exposure. Not for use in humans. Keep out of the reach of children. The ractopamine hydrochloride formulation (Type A Medicated Article) poses a low dust potential under usual conditions of handling and mixing. When mixing and handling ractopamine hydrochloride, use protective clothing, impervious gloves, protective eye wear, and a NIOSH-approved dust mask. Operators should wash thoroughly with soap and water after handling. If accidental eye contact occurs, immediately rinse eyes thoroughly with water. If irritation persists, seek medical attention. The material safety data sheet contains more detailed occupational safety information. To report adverse effects, access medical information, or obtain additional product information, call 1-800-428-4441.

MANUFACTURED BY

BLUE BIRD FEED MILL
Any town, USA 12345

Expiration Date: [30 days after manufacture]

07 January 2008

Date of Approval: February 15, 2008

FREEDOM OF INFORMATION SUMMARY

ORIGINAL NEW ANIMAL DRUG APPLICATION

NADA 141-278

ZILMAX plus RUMENSIN

(Zilpaterol Hydrochloride and Monensin USP)

Type A Medicated Articles
For Use in the Manufacture of Type B and C Medicated Feed
Cattle Fed in Confinement for Slaughter

For increased rate of weight gain, improved feed efficiency, increased carcass leanness, and prevention and control of coccidiosis due to *Eimeria bovis* and *E. zuernii* in cattle fed in confinement for slaughter for the last 20 to 40 days on feed.

Sponsored by:

Intervet Inc.

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I. GENERAL INFORMATION:

- A. File Number:** NADA 141-278
- B. Sponsor:** Intervet Inc.
P.O. Box 318
29160 Intervet Lane
Millsboro, DE 19966
Drug Labeler Code: 057926
- C. Proprietary Names:** ZILMAX plus RUMENSIN
- D. Established Names:** Zilpaterol hydrochloride and monensin USP
- E. Pharmacological Category:** Zilpaterol hydrochloride – Beta adrenergic agonist
Monensin USP – Ionophore/anticoccidial
- F. Dosage Forms:** Type A medicated articles to be used in the manufacture of Type B and C medicated feeds
- G. Amount of Active Ingredients:** Zilpaterol hydrochloride - 21.77 grams per pound (48 grams per kilogram)
Monensin USP – 80 grams per pound
- H. How Supplied:** Zilpaterol hydrochloride – 22.05 lb (10 kg) bag
Monensin USP – 50 lb bag
- I. How Dispensed:** OTC
- J. Dosages:** Zilpaterol is fed at a concentration of 6.8 g of zilpaterol hydrochloride per ton of complete feed to provide 60 to 90 mg zilpaterol/head/day for increased rate of weight gain, improved feed efficiency, and increased carcass leanness in cattle fed in confinement for slaughter during the last 20 to 40 days on feed.
- Monensin is added to diets for cattle fed in confinement for slaughter at concentrations of 10 to 40 g of monensin USP per ton of complete feed at a rate of 0.14 to 0.42 mg monensin/lb of body weight, depending on severity of coccidiosis challenge, up to 480 mg

monensin/head/day.

K. Route of Administration:

Oral, in feed

L. Species/Class:

Cattle fed in confinement for slaughter

M. Indication:

For increased rate of weight gain, improved feed efficiency, increased carcass leanness, and prevention and control of coccidiosis due to *Eimeria bovis* and *E. zuernii* in cattle fed in confinement for slaughter for the last 20 to 40 days on feed.

II. EFFECTIVENESS:

In accordance with the Federal Food, Drug, and Cosmetic Act (FFDCA), as amended by the Animal Drug Availability Act of 1996, if the animal drugs/active ingredients intended for use in combination in animal feed have previously been separately approved for the particular uses and conditions of use for which they are intended for use in combination, FDA will not refuse to approve an NADA for the combination on effectiveness grounds unless the FDA finds that the sponsor fails to demonstrate that:

- there is substantial evidence to indicate that any active ingredient/drug intended only for the same use as another active ingredient/animal drug in combination makes a contribution to the labeled effectiveness.
- each of the active ingredients or animal drugs intended for at least one use that is different from all other active ingredients or animal drugs used in the combination provides appropriate concurrent use for the intended target population.
- where the combination contains more than one nontopical antibacterial active ingredient/animal drug, there is a substantial evidence that each of the nontopical antibacterial active ingredients/animal drugs makes a contribution to the labeled effectiveness

Zilpaterol hydrochloride as provided by Intervet Inc., has previously been separately approved for use in cattle for increased rate of weight gain, improved feed efficiency, and increased carcass leanness in cattle fed in confinement for slaughter during the last 20 to 40 days on feed (21 CFR 558.665(e)(2)). Monensin USP, as provided by Elanco Animal Health, has previously been separately approved (in a supplemental approval dated December 1, 2006) for use in cattle fed in confinement for slaughter for prevention and control of coccidiosis due to *Eimeria bovis* and *E. zuernii* (21 CFR 558.355(f)(3)(vii)(a)). Effectiveness of each drug, zilpaterol hydrochloride and monensin USP, when administered alone in accordance with its approved uses and conditions of use, is demonstrated in Intervet Inc.'s approved NADA 141-258 for zilpaterol hydrochloride,

and Elanco Animal Health's NADA 095-735 for monensin USP, to which Intervet Inc. has right of reference.

Zilpaterol hydrochloride and monensin USP are each intended for a different use therefore the NADA need not demonstrate, by substantial evidence, that zilpaterol hydrochloride or monensin USP, contributes to the labeled effectiveness of the combination. Zilpaterol hydrochloride and monensin USP provide appropriate concurrent use because these drugs are intended to treat different conditions likely to occur simultaneously in cattle fed in confinement for slaughter during the last 20 to 40 days on feed. Zilpaterol hydrochloride is approved for increased rate of weight gain, improved feed efficiency, and increased carcass leanness. Monensin USP is approved for prevention and control of coccidiosis due to *Eimeria bovis* and *E. zuernii*.

III. TARGET ANIMAL SAFETY:

In accordance with the FFDCAs, as amended by the Animal Drug Availability Act of 1996, if the animal drugs/active ingredients intended for use in combination in animal feed have previously been approved separately for the particular uses and conditions of use for which they are intended for use in combination, FDA will not refuse to approve an NADA for the combination on target animal safety grounds unless

- there is a substantiated scientific issue specific to an active ingredient or animal drug used in the combination that cannot adequately be evaluated based on the information contained in the application for the combination, and FDA finds that the application fails to show that the combination is safe, or
- there is a scientific issue raised by target animal observations contained in the studies submitted to the NADA for the combination, and FDA finds that the application fails to show that the combination is safe.

Zilpaterol hydrochloride, as provided by Intervet Inc., has previously been separately approved for use in cattle for increased rate of weight gain, improved feed efficiency, and increased carcass leanness in cattle fed in confinement for slaughter during the last 20 to 40 days on feed (21 CFR 558.665(e)(2)). Monensin USP, as provided by Elanco Animal Health, has previously been separately approved (in a supplemental approval dated December 1, 2006) for use in cattle fed in confinement for slaughter for prevention and control of coccidiosis due to *Eimeria bovis* and *E. zuernii* (21 CFR 558.355(f)(3)(vii)(a)).

Under the provisions of ADAA, this original approval allows for the combination of zilpaterol hydrochloride (as provided by Intervet Inc.) and monensin USP (as provided by Elanco Animal Health). Target animal safety of each drug, zilpaterol hydrochloride and monensin USP, when administered alone in accordance with its approved uses and conditions of use, is demonstrated in Intervet Inc.'s approved NADA 141-258, and Elanco Animal Health's NADA 95-735, respectively. The Agency has found no substantiated scientific issue relating to the target animal safety of zilpaterol hydrochloride and monensin USP when used in combination under this NADA and no scientific issue has been raised by target animal observations submitted as part of the

NADA for this combination. Thus, pursuant to FFDCFA, as amended by the Animal Drug Availability Act of 1996, no specific target animal safety studies are required for approval of NADA 141-278.

IV. HUMAN FOOD SAFETY:

In accordance with the FFDCFA, as amended by the ADAA of 1996, if the animal drugs or active ingredients intended for use in combination in animal feed have already been separately approved for the particular uses and conditions of use for which they are intended for use in combination, CVM will not refuse to approve an NADA for the combination on human food safety grounds unless CVM finds that the application fails to establish that:

- none of the active ingredients or animal drugs used in combination at the longest withdrawal for any of the active ingredients or animal drugs in the combination exceeds the established tolerance, or
- none of the active ingredients or animal drugs in combination interferes with the method of analysis for another active ingredient or animal drug in the combination

A. Toxicology:

Safety of the individual drugs in this combination product has been established by data in NADA 141-258 for zilpaterol hydrochloride (FOI Summary dated August 10, 2006), and NADA 095-735 for monensin USP (FOI Summary dated December 1, 2006).

B. Residue Chemistry:

1. Summary of Residue Chemistry Studies

Data demonstrating residue depletion and assay noninterference for the drugs of this combination have been summarized in the FOI Summary for the approval of NADA 141-276 dated January 10, 2008.

2. Target Tissue and Marker Residue Assignment

The marker residue for zilpaterol is zilpaterol freebase and the target tissue in cattle is liver (NADA 141-258, *op. cit.*). No marker residue and target tissue is specified for monensin.

3. Tolerance Assignments

The tolerance for zilpaterol freebase is 12 ppb in cattle liver (21 CFR 556.765). The tolerances for monensin in cattle are 0.05 ppm for muscle, kidney and fat, and 0.10 ppm for liver (21 CFR 556.420).

4. Withdrawal Time(s)

The residue depletion data referred to in NADA 141-276 confirm that (1) residues of monensin in a 4 way combination including zilpaterol hydrochloride, monensin USP, tylosin phosphate, and melengestrol acetate at zero withdrawal period are less than applicable tolerances and (2) the statistically determined tolerance limit for residues of zilpaterol at 3 days of withdrawal is less than the tolerance of 12 ppb. These results support the assignment of a 3-day withdrawal period for zilpaterol hydrochloride when used in combination with monensin.

C. Microbial Food Safety:

The Agency determined that an assessment of the microbial food safety associated with this application for the combination of zilpaterol hydrochloride and monensin USP for use in cattle, approvable pursuant to the provisions of the Animal Drug Availability Act (1996), was not necessary at this time.

D. Analytical Method for Residues:

Refer to NADA 141-258 for zilpaterol (*op. cit.*) and to NADA 095-735 for monensin (*op. cit.*) for the approved regulatory methods. The methods are available from the Center for Veterinary Medicine, FDA, 7500 Standish Place, Rockville, MD 20855.

V. USER SAFETY:

The product labeling contains the following information regarding safety to humans handling, administering, or exposed to ZILMAX:

WARNING:

The active ingredient in Zilmax[®] is zilpaterol hydrochloride, a beta₂-adrenergic agonist. Not for use in humans. An anti-dust process has been applied to the drug product, Zilmax[®], in order to greatly reduce inhalation risk. Extended handling tasks with the potential for dust generation require respiratory protection. Wear appropriate skin protection (e.g., impervious gloves, apron, overalls) if there is a potential for extended skin contact. Wear protective eye wear, if there is a potential for eye contact. If accidental eye contact occurs, immediately rinse with water and consult a physician.

The representative (blue bird) labeling for the Type B and Type C medicated feeds contains no information regarding safety to humans handling, administering, or exposed to RUMENSIN. This is based upon review of the material safety data sheet (MSDS) for RUMENSIN, as well as the MSDS for ZILMAX, and the individually approved blue bird labeling.

VI. AGENCY CONCLUSIONS:

The data submitted in support of this NADA satisfy the requirements of section 512(d)(4) of the Federal Food, Drug, and Cosmetic Act and 21 CFR Part 514. The data demonstrate that ZILMAX plus RUMENSIN, when used according to the label, is safe and effective for increased rate of weight gain, improved feed efficiency, increased carcass leanness, and prevention and control of coccidiosis due to *Eimeria bovis* and *E. zuernii* in cattle fed in confinement for slaughter for the last 20 to 40 days on feed. Additionally, data demonstrate that residues in food products derived from cattle fed in confinement for slaughter treated with ZILMAX plus RUMENSIN will not represent a public health concern when the product is used according to the label.

The drugs are to be fed in Type C medicated feeds in accordance with section II and III of the FOI Summary and the Blue Bird labeling that is attached to this document.

A. Marketing Status:

The Center for Veterinary Medicine has concluded that, for this product, adequate directions for use by the lay person have been provided. Label directions provide detailed instruction in plain language. The drug product is not a controlled substance. Thus, the drug product is assigned OTC status, and the labeling is adequate for the intended use.

B. Exclusivity:

This approval does not qualify for marketing exclusivity under section 512(c)(2)(F)(ii) of the Federal Food, Drug, and Cosmetic Act.

C. Patent Information:

ZILMAX is under the following US patent numbers:

<u>U.S. Patent Number</u>	<u>Date of Expiration</u>
4,900,735	December 11, 2008
5,731,028	June 6, 2016
7,207, 289	May 20, 2025

VII. ATTACHMENTS:

Final Printed Labeling:

Zilpaterol and Monensin Type B Medicated Cattle Feed
Zilpaterol and Monensin Liquid Type B Medicated Cattle Feed
Zilpaterol and Monensin Type C Medicated Cattle Feed

PubMed

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Performing your original search, *ractopamine horses*, in PubMed will retrieve [4 records](#).



[J Anal Toxicol](#). 2004 May-Jun;28(4):226-38.

Detection and confirmation of ractopamine and its metabolites in horse urine after Paylean administration.

[Lehner AF](#), [Hughes CG](#), [Harkins JD](#), [Nickerson C](#), [Mollett B](#), [Dirikolu L](#), [Bosken J](#), [Camargo F](#), [Boyles J](#), [Troppmann A](#), [Karpiesiuk WW](#), [Woods WE](#), [Tobin T](#).

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Abstract

We have investigated the detection, confirmation, and metabolism of the beta-adrenergic agonist ractopamine administered as Paylean to the horse. A Testing Components Corporation enzyme-linked immunosorbent assay (ELISA) kit for ractopamine displayed linear response between 1.0 and 100 ng/mL with an I-50 of 10 ng/mL and an effective screening limit of detection of 50 ng/mL. The kit was readily able to detect ractopamine equivalents in unhydrolyzed urine up to 24 h following a 300-mg oral dose. Gas chromatography-mass spectrometry (GC-MS) confirmation comprised glucuronidase treatment, solid-phase extraction, and trimethylsilyl derivatization, with selected-ion monitoring of ractopamine-tris(trimethylsilane) (TMS) m/z 267, 250, 179, and 502 ions. Quantitation was elaborated in comparison to a 445 Mw isoxsuprine-bis(TMS) internal standard monitored simultaneously. The instrumental limit of detection, defined as that number of ng on column for which signal-to-noise ratios for one or more diagnostic ions fell below a value of three, was 0.1 ng, corresponding to roughly 5 ng/mL in matrix. Based on the quantitation ions for ractopamine standards extracted from urine, standard curves showed a linear response for ractopamine concentrations between 10 and 100 ng/mL with a correlation coefficient $r > 0.99$, whereas standards in the concentration range of 10-1000 ng/mL were fit to a second-order regression curve with $r > 0.99$. The lower limit of detection for ractopamine in urine, defined as the lowest concentration at which the identity of ractopamine could be confirmed by comparison of diagnostic MS ion ratios, ranged between 25 and 50 ng/mL. Urine concentration of parent ractopamine 24 h post-dose was measured at 360 ng/mL by GC-MS after oral administration of 300 mg. Urinary metabolites were identified by electrospray ionization (+) tandem quadrupole mass spectrometry and were shown to include glucuronide, methyl, and mixed methyl-glucuronide conjugates. We also considered the possibility that an unusual conjugate added 113 amu to give an observed m/z 415 [M+H] species or two times 113 amu to give an m/z 528 [M+H] species with a daughter ion mass spectrum related to the previous one. Sulfate and mixed methyl-sulfate conjugates were revealed following glucuronidase treatment, suggesting that sulfation occurs in combination with glucuronidation. We noted a paired chromatographic peak phenomenon of apparent ractopamine metabolites appearing as doublets of equivalent intensity with nearly identical mass spectra on GC-MS and concluded that this phenomenon is consistent with Paylean being a mixture of RR, RS, SR, and SS diastereomers of ractopamine. The results suggest that ELISA-based screening followed by glucuronide hydrolysis, parent drug recovery, and TMS derivatization provide an effective pathway for detection and GC-MS confirmation of ractopamine in equine urine.

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