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CLEARANCE TIMES AND THE FORENSIC SIGNIFICANCE OF THE DIETARY ANTHELMINTIC PYRANTEL TARTRATE IN PERFORMANCE HORSES

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SUMMARY

Six performance bred (Thoroughbred or Standardbred) mares were fed the anthelmintic pyrantel tartrate as a daily supplement for a period of 21 days to assure steady state concentrations would be achieved. The forensic "clearance times" and potential for analytical interference of pyrantel tartrate were then investigated. This investigation was intended to enable guidelines to be established for veterinarians and trainers to avoid a "positive" test result for pyrantel which might violate existing rules or regulations.

The analysis of blood and urine samples from these horses was conducted by the University of Kentucky Equine Drug Testing Laboratory, and were performed on samples obtained on days -3, -2, -1, 7, 14, 21, +1, +2, +3 and +4. Pyrantel tartrate was readily detected by standard thin layer chromatography analysis in urine samples on days 7, 14 and 21 during the administration portion of the study, and on day +1 of the post-administration time period. Further analysis of the positive urine samples by direct probe mass spectrometry confirmed the presence of pyrantel. In our analysis of equine serum samples pyrantel tartrate was not detected.

Pyrantel positive urine samples were additionally analyzed using enzyme-linked immunosorbent assays designed to detect fluphenazine, butorphanol, morphine, oxymorphone, fentanyl, sufentanil and etorphine, with no cross-reactivity found. We concluded that, from a drug testing perspective, the use of pyrantel is unlikely to interfere with normal post-race drug testing.

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INTRODUCTION

In most equine deworming programs the horse is administered an anthelmintic or similar deworming medication every 30 to 60 days depending on the particular program or the manager's preference. The anthelmintic pyrantel tartrate however, is designed to be supplemented in the feed on a daily basis.[9] This daily treatment schedule is believed to aid the horse by substantially lowering the day to day intestinal population of parasites.[1,2,3] In fact, this type of treatment regimen has been shown to significantly decrease the intestinal penetration of infective *Strongylus Vulgaris* larva, and additionally to eliminate adult forms of large and small strongyles, *Parascaris Equorum*, and *Oxyuris Equi*.[4,5,8]

While most racing or show horse jurisdictions do not forbid equine athletes from competing with anthelmintics in their systems, the use of these medications are regulated in

some instances. Problems can arise, therefore, if the veterinarian or trainer is not aware of the "clearance time" needed for a horse to be forensically negative for a particular medication. Forensically negative in this context is defined as an absence of any discernible material from a sample on thin layer chromatography (TLC) plate following standard extraction procedures.

In order to obtain samples for analysis of the forensic significance of pyrantel tartrate, six performance bred mares were fed pyrantel tartrate as a feed supplement for 21 days to assure that steady state concentrations would be reached. At the end of this time period, the mares were sampled daily until determined to be forensically negative for three consecutive days by the equine drug testing laboratory at the University of Kentucky. The analysis was performed by subjecting the samples to the routine screening procedures used by the equine drug testing lab in their normal post-race testing programs. Prior to analysis, the samples were subjected to enzyme hydrolysis, and both acid and base extraction procedures. A series of chromatographic analyses were conducted to determine a standard R_f (migration fraction on a TLC plate) value for pyrantel tartrate for comparison purposes. Beyond this, an extraction procedure was developed specifically for pyrantel tartrate for the purpose of maximizing its detection in equine urine samples. This procedure was developed as an aid in ensuring that the horses were forensically negative, and to aid in the further analysis of these samples by gas chromatography-mass spectrometry (GC-MS).

In addition to the analytical detection of pyrantel, there is little information as to the potential of pyrantel tartrate to interfere with the analytical detection of drugs of abuse in racing horses. To that end, urine samples determined to be positive for pyrantel by TLC, as well as negative control samples, were included with other equine urine samples analyzed by enzyme-linked immunosorbent assay (ELISA) tests to investigate the possible cross reactivity of pyrantel in these tests. The tests used were designed to detect butorphanol, fluphenazine, fentanyl, morphine, oxymorphone, sufentanil, and etorphine.

MATERIALS AND METHODS

Horses

Six purebred (Thoroughbred or Standardbred) mares that were routinely maintained at pasture at the University of Kentucky's equine research farm were used in the study. Individual data on the horses is illustrated in Table 1. The horses were brought daily to the research barn and placed in individual box stalls with free access to hay and water. After blood and urine samples were obtained, the mares were fed either the control feed or the control feed plus the pyrantel tartrate. The horses were allowed approximately 2 hours to consume the feed, at which time they were returned to the research pasture.

Table 1.

Horse	Breed	Weight (kg)	Dose* (gm/day)
A	Standardbred	570	14.1
B	Thoroughbred	480	11.3
C	Thoroughbred	490	12.2
D	Thoroughbred	485	12.2
E	Thoroughbred	545	13.2
F	Standardbred	545	13.2

*Daily dosage of premix containing 11% pyrantel tartrate. Actual pyrantel tartrate dose = 2.64 mg/kg body weight.

Feed mixture

The control feed mixture consisted of approximately 100 gm of clean dry oats, 20 ml of liquid molasses, and approximately 300 gm of a sweet feed ration.[a] The test ration contained, in addition to the control mixture, 2.6 mg of pyrantel tartrate (Strongid C)[b] per kg body weight. Strongid C is a pre-mixed feed supplement that contains approximately 11% pyrantel tartrate by weight.

Experimental protocol

The horses were brought daily to the research barn and acclimated to control conditions (control feed mixture, handling and sampling procedures) for a period of 7 days. They were then fed pyrantel tartrate[b] at the manufacturers recommended dose rate for 21 days. At the end of this administration period, they were maintained on the control feed until determined forensically negative for pyrantel for 3 consecutive days. Serum samples were obtained by venipuncture from the left side of the horse, while urine samples were obtained by bladder catherization. Samples were obtained during the predose period (days -3, -2, -1), the treatment period (7, 14, 21), and the post-dose period (+1, +2, +3, +4, +5, and +6).

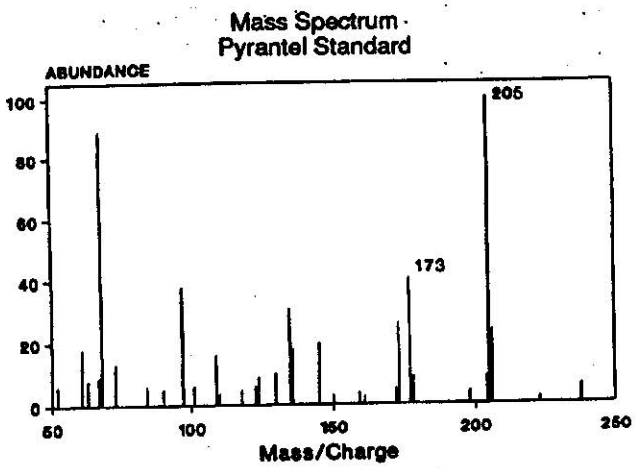
Analytical procedures

Urine samples were subjected to the "routine drug screen" as performed by the Kentucky Equine Drug Testing Laboratory for its testing of post-race samples. The methods used included: enzyme hydrolysis, base, acid and neutral liquid-liquid extraction procedures, and a "special" base extraction method optimized for detection of pyrantel, all followed by thin layer chromatography (TLC). In addition, selected samples were analyzed by direct probe mass spectrometry and enzyme linked immunosorbent assays (ELISA).

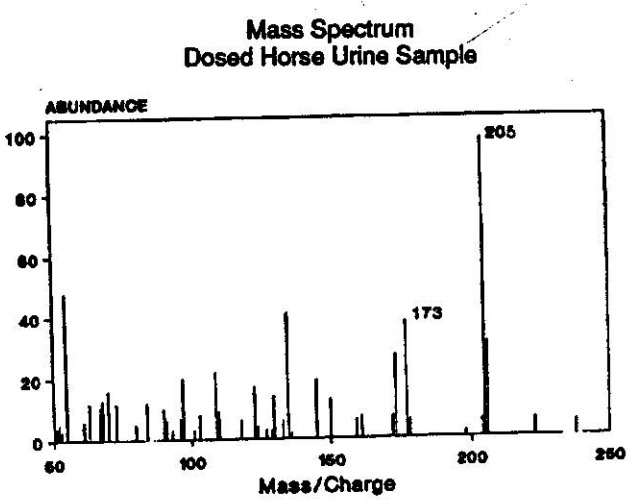
Enzyme hydrolysis

Enzyme hydrolysis was accomplished by first adding 1 ml (5000 units) of beta-glucuronidase, 2 ml of pH 5 acetate buffer, and 5 ml of urine sample to a clean glass tube. The mixture was then heated for 3 hours at 65°C, and then allowed to cool to room temperature.

[a] Omalene 100, Purina Co.
[b] Pfizer Inc., Lees Summit, MO



(A) Pyrantel tartrate drug standard mass spectra.



(B) Day 14 equine urine sample mass spectra.

Figure 1. Mass spectra of pyrantel tartrate (A), and mass spectra from the extract of an equine urine sample obtained on Day 14 of the treatment period (B).

When horse urine samples were analyzed after the direct addition of pyrantel to the sample, the analysis consistently showed a spot at a slightly greater R^f than that seen for the pyrantel standard. However, after re-chromatography and mass spectral analysis of the material isolated from these plates, the material was shown to be identical with pyrantel. We determined that this material producing the second spots was probably the cis isomer, since the trans isomer is readily photoisomerized.[6]

The TLC screening methods applied to samples obtained from the horses during the treatment period (days 7, 14, and 21) did not reveal any unusual components, and thus appeared to be identical with the corresponding control samples with the exception of the spot identified as pyrantel.

As stated above, the urine samples from the treatment period were shown to be positive for pyrantel by TLC, and the presence of pyrantel in these samples was readily confirmed by direct probe mass spectrometry. The use of a direct probe or liquid chomatographic interface in a mass spectral analysis of pyrantel is recommended since pyrantel is poorly eluted from methyl silicone gas chromatography columns.[6,10]

Table 3. Analysis of pyrantel positive urine samples by ELISA.

Horse	A	B	C	D	E	F
ELISA (drug)						
etorphine	107	92	106	113	101	109
morphine	96	82	100	92	82	88
sufentanil	110	88	116	13	95	95
oxymorphone	109	85	104	112	88	95
fluphenazine	85	88	94	96	88	95
fentanyl	94	84	121	93	87	84
butorphanol	98	92	96	118	90	95

Values given are % of control optical densities determined from pre-administration urine samples.

A comparison of the mass spectral data generated from samples of pyrantel tartrate drug standard, and that from urine samples subjected to preparative TLC is given in Figure 1. The identifiable pyrantel mass/charge peaks at 205 and 173 are seen in both spectrums indicating the presence of pyrantel in the dosed horse urine sample.

The urine samples from day +1, which were collected exactly 24 hours after the last dose, showed a much diminished spot for pyrantel as compared to the samples from the treatment period. Attempts at mass spectrometric confirmation of the day +1 samples resulted in poor quality spectra. Therefore, many samples from this time period would probably be labeled "suspicious" rather than "positive" in a post-race drug testing setting.

We did not identify any major urinary metabolites in our TLC screening methods, nor in our mass spectral analysis. However, possible hydroxylated metabolites would most likely be difficult to distinguish from pyrantel in the base extract/Davidow TLC system. In addition, potential degradation products such as thiophene-acrylic acids or N-methyl propanediamine, if detected, would probably be viewed as normal horse urine constituents, and not be associated with drug use by a racing analyst.[6,7]

Urine samples collected two or more days after the last dose were all negative by TLC indicating that pyrantel is rapidly cleared, or at least its detection time is limited.

The results of our analysis of the potential of pyrantel to interfere with, or more accurately, cross-react with several immunoassay tests now employed by many racing jurisdictions are presented in Table 3. The optical densities of the horses control urine samples were used for comparison purposes, and the results show that the pyrantel positive samples were all read as essentially negative (between 80 and 120%) in the ELISA screening tests. Optical densities in the range of 80 to 120% of control values fall within the range of normal background levels in equine urine samples.[11] Based on these results, we feel that the likelihood of any appreciable cross reactivity by pyrantel in immunoassay based testing is remote.