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Journal

Journal of Veterinary Diagnostic Investigation, 34(3)

ISSN

1040-6387

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Publication Date

2022-05-01

DOI

10.1177/10406387221086923

Peer reviewed

Journal of Veterinary Diagnostic Investigation
2022, Vol. 34(3) 489–495

DOI: 10.1177/10406387221086923
jvdi.sagepub.com

Determining an approximate minimum toxic dosage of diphacinone in horses and corresponding serum, blood, and liver diphacinone concentrations: a pilot study

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Abstract. Poisoning of nontarget species is a major concern with the use of anticoagulant rodenticides (ARs). At postmortem examination, differentiating toxicosis from incidental exposure is sometimes difficult. Clotting profiles cannot be performed on postmortem samples, and clinically significant serum, blood, and liver AR concentrations are not well-established in most species. We chose diphacinone for our study because, at the time, it was the publicly available AR most commonly detected in samples analyzed at the University of Kentucky Veterinary Diagnostic Laboratory. We determined an approximate minimum toxic dosage (MTD) of oral diphacinone in 3 horses and measured corresponding serum, blood, and liver diphacinone concentrations. Diphacinone was administered orally to healthy horses. Prothrombin time (PT), activated partial thromboplastin time (aPTT), and serum and blood diphacinone concentrations were measured daily. At the study endpoint, the horses were euthanized, and diphacinone concentration was measured in each liver lobe. The horse that received 0.2 mg/kg diphacinone developed prolonged (>1.5× baseline) PT and aPTT; the horse that received 0.1 mg/kg did not. This suggests an approximate oral MTD in horses of 0.2 mg/kg diphacinone. Median liver diphacinone concentration at this dosage was 1,780 (range: 1,590–2,000) ppb wet weight. Marginal (model-adjusted) mean diphacinone concentrations of liver lobes were not significantly different from one another (p = NS). Diphacinone was present in similar concentrations in both serum and blood at each time after administration, indicating that both matrices are suitable for detection of diphacinone exposure in horses.

Keywords: anticoagulants; diphacinone; horses; pesticides; rodenticides.

Anticoagulant rodenticides (ARs) have been used for many years to control rodent populations on agricultural and residential properties, including horse farms. Although effective at controlling some rodent populations, ARs can pose a risk to horses and other nontarget species. Apple-flavored products and grain-based pelleted formulations may be especially palatable to horses. ARs inhibit vitamin K epoxide reductase, an enzyme necessary for conversion of vitamin K epoxide to the hydroquinone form.[10,14,15] Active vitamin K hydroquinone is required for post-translational carboxylation of clotting factors II, VII, IX, and X (FII, FVII, FIX, FX), known as the vitamin K–dependent clotting factors. Carboxylation is essential for assembly of functional coagulation complexes; its inhibition causes a combined deficiency of vitamin K–dependent clotting factors, and ultimately, coagulopathy.

Clinical signs of AR poisoning depend on the location and severity of hemorrhage and can range from mild lethargy to acute collapse and death. The onset of action is typically delayed 48 h or longer after AR exposure, but can vary depending on the active ingredient, dosage, product formulation, single versus repeated exposures, endogenous levels of clotting factors and vitamin K, dietary vitamin K intake, and other factors. In horses, strenuous exercise may lower the toxic threshold.[2,4] Current analytical methods for AR detection in blood and tissues can quantify many ARs down to low ppb concentrations, but distinguishing clinical toxicosis from incidental exposure can be difficult. AR concentrations associated with coagulation abnormalities have not been

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determined in horses, nor have clinically relevant blood or tissue concentrations, to our knowledge.

Diphacinone was the AR most commonly detected in samples analyzed at the University of Kentucky Veterinary Diagnostic Laboratory (Lexington, KY, USA) at the time of our study. The primary goals of our pilot study were to determine an approximate minimum toxic dosage (MTD) of diphacinone in horses and to characterize the corresponding whole blood, serum, and liver diphacinone concentrations. We defined MTD as the lowest single dosage that caused coagulopathy. Additional study objectives included assessing suitability of whole blood and serum for antemortem diphacinone analysis and characterizing postmortem hepatic diphacinone distribution to provide guidance for sample collection in potential diphacinone exposure cases.

Materials and methods

Animals

All study procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Kentucky, pursuant to the requirements of the Animal Welfare Act.[1] Three healthy adult Thoroughbred mares were selected from among University of Kentucky research horses scheduled for euthanasia for reproductive, behavioral, or other non–health-related issues. Physical examinations, CBC, serum chemistry panels, prothrombin time (PT), activated partial thromboplastin time (aPTT), and blood and serum AR analyses were performed on all horses. Horses were excluded if there was evidence of systemic illness or abnormal laboratory assessments. The horses were held in a mixed-grass pasture and fed supplemental grass hay and a pelleted vitamin and mineral supplement. At least 5 d prior to diphacinone administration, the selected horses were moved to smaller paddocks to facilitate catching and handling. The horses were maintained on their normal diet and acclimated to sweet feed by offering ~0.5 kg of sweet feed daily.

Study design

Diphacinone was mixed with ~0.5 kg of sweet feed and offered as a single oral administration. Horse 1 was administered ~0.4 mg/kg body weight technical-grade diphacinone powder (MilliporeSigma). The technical-grade diphacinone powder proved difficult to dose accurately. Therefore, we administered liquid diphacinone sodium bait (Liqua-Tox II; Bell Laboratories) to horse 2 (0.2 mg/kg) and horse 3 (0.1 mg/kg).

Horses were examined 2 or 3 times per day after diphacinone administration. Physical examinations and blood sample collections were performed at ~9:00 am daily; physical examinations were repeated in the afternoon and in the evening. Samples included citrated plasma for coagulation assays (PT and aPTT) and serum and whole blood for diphacinone quantification. Whole blood was also collected 1–2 h prior to euthanasia for repeat CBCs. Early termination criteria included significant prolongation of PT and aPTT (>2× baseline); clinically evident coagulopathy (e.g., hematoma, excessive bleeding from venipuncture sites, epistaxis); nonspecific clinical signs that could be associated with occult hemorrhage (e.g., pallor, lethargy, elevated heart or respiratory rate, abdominal discomfort, neurologic abnormalities); and evidence of distress or severe clinical signs of any kind (e.g., dyspnea, seizures, coma, collapse). The horses were euthanized, and postmortem examinations were performed. Liver samples were collected from each lobe (quadrate, caudate, left medial, left lateral, and right lobes) and frozen at −20°C for diphacinone analysis.

Coagulation assays

Immediately after sample collection, coagulation assays were performed at a nearby laboratory (Rood and Riddle Equine Hospital Laboratory, Lexington, KY, USA) to facilitate early recognition of significantly prolonged PT and aPTT necessitating early study termination. "Significant prolongation" was defined as an increase >2× baseline. For horse 1, the initial coagulation profile served as the baseline. For horses 2 and 3, coagulation profiles were repeated for several days prior to administration (–3 d, –2 d, –1 d, and 0 d), and mean baseline values were calculated.

Additional aliquots of citrated plasma from horses 2 and 3 were stored at −20°C and shipped on dry ice to the Comparative Coagulation Laboratory at Cornell University (CCCL; Ithaca, NY, USA). The samples were stored at −70°C until thawed at 37°C immediately before batch assay of PT and aPTT. The PT was performed with a rabbit brain thromboplastin reagent (Thromboplastin LI; Helena Diagnostics), and the aPTT was performed with a 3-min activation time and an ellagic acid–soy phosphatide reagent (Actin FS; Siemens). The PT international normalized ratio (PT-INR) was calculated from the ratio of patient PT to the PT assay mean and the PT reagent sensitivity index.[8] The ratio of patient aPTT to a same-day assay control value (aPTT P:C ratio) was also calculated.

Diphacinone analysis

Serum, whole blood, and liver samples were analyzed for diphacinone using liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods.[13] All reagents and chemicals were HPLC grade and/or >97% purity unless otherwise noted. Chromatographic separation was conducted with 100 × 2.1 mm, 2.6-µm analytical columns (Accucore C18; Thermo Fisher). Mobile phase A consisted of 10 mM ammonium acetate in water adjusted to pH 9; mobile phase B consisted of methanol. The mass spectrometers utilized electrospray ionization (ESI) sources in negative ion mode. For diphacinone, the deprotonated molecular ion at *m/z* 339

was used as the precursor ion, with product ions at *m/z* 167 (quantification) and *m/z* 145 (qualifier). For d4-diphacinone (the internal standard for the serum and whole blood analyses), the precursor ion was *m/z* 343, and the sole product ion monitored was *m/z* 167.

Serum and whole blood

Serum and whole blood samples were analyzed for diphacinone using isotope-dilution high-performance LC-MS/MS. Briefly, each 0.25-g aliquot was extracted 3 times sequentially with 0.25-mL aliquots of cold 10% Optima-grade acetone in methanol (Thermo Fisher). Calibrants were produced by fortifying negative control equine serum with diphacinone (certified >99% pure; MilliporeSigma) and then extracting as for diagnostic samples. Prior to extraction, all samples and calibrants were fortified with 50-ppb d4-diphacinone internal standard (certified 99% pure; CDN Isotopes).

The extracts were filtered through a 0.22-µm syringe filter and analyzed using a 1290 ultra-high-performance liquid chromatography (UHPLC) system interfaced with a model 6460 triple-stage quadrupole mass spectrometer and Jet-Stream ESI source (Agilent). The mobile-phase gradient started at 20% B, was held for 1 min, then was ramped to 95% B at 10 min. It was held at 95% B until 15 min, then brought back to 20% B and held until 21 min to re-equilibrate the column.

Calibration curves were constructed by analyzing serum calibrants fortified at 5 different concentrations of diphacinone between 1.0 and 100 ppb. The method was validated as per FDA-CVM guidelines for Level 1 method validation.[6] The minimum level of detection (MLD) of 0.2 ppb was determined using the EPA procedure outlined in 40 CFR Appendix B.[5] The minimum level of quantification (MLQ) was set at 1.0 ppb, the lowest point of the calibration curve. Concentrations above the MLD but below the MLQ were designated "trace."

Liver

Samples of each liver lobe were analyzed for diphacinone using a previously described LC-MS/MS method.[13] Briefly, 1-g subsamples were minced and homogenized in a 50-mL polypropylene centrifuge tube with a 9.5-mm stainless steel ball bearing (2010 Geno/Grinder; SPEX SamplePrep) for 5 min at 650 strokes per minute. Homogenates were extracted in 6 mL of 10% methanol in acetonitrile on a reciprocating shaker (MaxQ 2500; Thermo Fisher) for 30 min. The tubes were centrifuged at 1,800 × *g* for 5 min (Sorvall Legend RT+; Thermo Scientific), and the extracts were transferred to dispersive–solid phase extraction (d-SPE) tubes containing 175 mg of MgSO4, 100 mg of Florisil, 50 mg of alumina basic, and 50 mg of primary secondary amine (UCT Technologies). The tubes were then vortexed, rotated on a tube mixer for 30 min, and centrifuged for 5 min at 1,800 × *g*. Each supernatant was decanted into a 15-mL polypropylene centrifuge tube and evaporated to dryness at 45°C (XcelVap, Horizon Technology; and/or N-EVAP III, Organomation). Residues were reconstituted in 1 mL of LC/MS-grade methanol, vortexed, and sonicated (FS-30; Fisher Scientific) for 5 min. Extracts were filtered through 13-mm diameter 0.22-µm polytetrafluoroethylene syringe filters (Restek) into silanized glass autosampler vials. Calibrants were produced by fortifying negative ovine control liver with diphacinone (certified >98.1% pure; US EPA National Pesticide Repository) and treating as diagnostic samples.

Extracts were analyzed using an UltiMate 3000 UHPLC system (Thermo Fisher Dionex) interfaced with a TSQ Quantum Access Max mass spectrometer and heated ESI (Thermo Scientific). The mobile-phase gradient started at 40% B, was maintained for 1 min, increased to 57% over the next 8 min, and increased further to 77% over 6 min. Mobile phase B was increased to 81% over the next 3 min, further increased to 90% over 1 min, then held at 90% from 19–24 min post-injection. Initial mobile phase conditions were resumed for column re-equilibration. The total run time for one injection was 34 min. Calibration curves were constructed by analyzing liver fortified at 7 different concentrations of diphacinone between 25 and 2,500 ppb. The MLD (25 ppb) and MLQ (50 ppb) were established previously.[13] As with the serum and whole blood method, concentrations between the MLD and MLQ were designated "trace."

Statistical analysis

All statistical analyses were conducted with Stata 16.1MP (StataCorp), with 2-sided tests of hypotheses and $p \leq 0.05$ as the criterion for statistical significance. Descriptive statistics were reported as medians and ranges of continuous variables. Test of normal distribution (Shapiro–Wilk test) was performed to determine extent of skewness of data. For the purpose of inference statistical analysis, mixed-effects (ME) linear regression was used for analysis of the outcome of interest, diphacinone concentration. Fixed effects were set as the dosage and the liver lobe. Random effects were set on the level of the dosage nested within the individual animal. Post-hoc pairwise calculations were conducted to estimate marginal (model-adjusted) means and differences. To adjust for multiple comparisons, the least significant difference method was used.

Results

All horses remained bright, alert, and responsive for the duration of our study. Physical examination findings, including heart and respiratory rate, pulse quality, and capillary refill time remained within normal limits. No clinically evident coagulopathy was observed in any of the horses, and early study terminations were not necessary. No gross or histologic evidence of hemorrhage was present on postmortem examination.

Coagulation assays

Horse 1 blood samples were assayed at the local laboratory only. Blood samples from horses 2 and 3 were assayed at both the local and referral laboratories. Prior to diphacinone administration, PT and aPTT were within normal RIs for all 3 horses (Tables 1 and 2). Horses 1 and 2 developed prolonged (>1.5× baseline) aPTT by 48 h after diphacinone administration. Horse 2 also developed slightly prolonged PT (outside the RI, but <1.5× baseline) and elevated PT-INR (1.5× baseline) by 3 d after administration (Tables 1, 2). Horse 3 maintained normal PT and aPTT throughout our study.

Diphacinone analysis

Diphacinone was not detected in pre-administration blood and serum samples of any horse (MLD 0.2 ppb). At 24 h, serum concentrations were 340, 92, and 9.6 ppb in horses 1–3, respectively; corresponding whole blood concentrations were 310, 160, and 28 ppb (Fig. 1). Concentrations decreased at each subsequent time but remained quantifiable (MLQ 1.0 ppb) through the study endpoints. Final serum concentrations were as follows: horse 1, 42 ppb (3 d); horse 2, 8.0 ppb (4 d); and horse 3, 3.7 ppb (4 d). Final whole blood concentrations were similar.

Diphacinone was detected in all liver lobes of all 3 horses at postmortem examination (Fig. 2). Median liver lobe diphacinone concentrations in horses 1–3 were 2,290 (range: 1,840–2,470) ppb wet weight (ww); 1,780 (range: 1,590–2,000) ppb ww; and 1,600 (range: 1,430–1,820) ppb ww, respectively (Table 3). Marginal mean diphacinone concentrations by liver lobe ranged from 1,800 ppb ww (95% CI: 1,680–1,920; caudate lobe) to 1,920 ppb ww (95% CI: 1,810–2,040; left lateral lobe; Table 3). No significant differences (p = NS) were noted between marginal mean liver concentrations of any lobe.

Discussion

Our primary objective for this project was to estimate a minimum toxic dosage for diphacinone in horses. Published minimum toxic dosages in dogs are 3.0–7.5 mg/kg.[11] We used the lower end of this range (4.5 mg/kg) as a starting point and used one-tenth the dosage (0.45 mg/kg) to allow for possible increased sensitivity in horses. Our administration protocol was designed to mimic field conditions of diphacinone intoxication as closely as possible.

The original protocol was to offer ground diphacinone-containing bait mixed with sweet feed. Most block and pellet formulations contain 0.005% diphacinone. A dosage of 0.4 mg/kg would require ~4 kg of bait for a 500-kg horse. The bait material used initially was a waxy weatherproof block formulation, which did not mix well into the sweet feed even when broken into smaller pieces. Horse 1 refused to eat the mixture despite attempts to make it more palatable by adding pancake syrup. We then mixed technical-grade diphacinone powder with sweet feed, which the horse readily accepted. The advantages of using the technical-grade product included the very small volume necessary to achieve our desired dosage, and the apparently minimal effect on palatability of the sweet feed. The major disadvantages were the poor solubility of the material and its tendency to adhere to surfaces, both of which precluded accurate calculation of the dosage the horse ultimately received. Allowing for transfer losses, horse 1 received ~0.4 mg/kg, but this is a rough estimate.

Table 1. Horse 1 (diphacinone 0.4 mg/kg) prothrombin time (PT) and activated partial thromboplastin time (aPTT) results from Rood and Riddle Equine Hospital.

Assay	Baseline	24 h	48 h	3 d	RI
PT (s)	11.4	10.2	14.9	10.0	10.0–18.0
aPTT (s)	38.9	48.3	59.5*	58.5*	30.0–60.0

* Indicates values ≥1.5× baseline.

For horses 2 and 3, we obtained a liquid bait containing diphacinone sodium salt (Liqua-Tox II; Bell Laboratories). The liquid formulation was much easier to handle and measure compared to technical-grade diphacinone, and both horses willingly ate the sweet feed mixed with the liquid. The liquid bait was 0.106% diphacinone sodium. A dosage of 0.2 mg/kg would require 94 mL of liquid bait for a 500-kg horse, and a dosage of 0.1 mg/kg would require 47 mL.

We defined prolongation of PT and aPTT as an increase ≥1.5× baseline. After reviewing the results for horse 1, we realized that by using a single coagulation profile as a baseline, this protocol did not account for potential day-to-day variation. Therefore, for horses 2 and 3, we modified the protocol to include 3 additional days of baseline coagulation profile measurements to better characterize normal inter-day variability. We then calculated mean baseline values with which to compare subsequent post-administration measurements. The post-administration monitoring period was also extended for an additional day to characterize antemortem blood and serum concentrations for a longer period post-exposure.

For horses 2 and 3, additional coagulation tests were performed at the CCCL. These included PT and aPTT assays and calculation of aPTT P:C ratio and PT-INR. The aPTT P:C ratio minimizes the influence of inter-assay variability by comparing values obtained from diagnostic samples with values obtained from control samples analyzed simultaneously under the same environmental conditions. The PT-INR data transformation is used in human medicine to standardize vitamin K–antagonist therapy. The PT-INR value, unlike absolute PT clotting times, is directly comparable across laboratories using different PT reagents, thereby enabling consistent dosage guidelines to attain a

Table 2. Horse 2 (diphacinone 0.2 mg/kg) and horse 3 (diphacinone 0.1 mg/kg) prothrombin time (PT), activated partial thromboplastin time (aPTT), PT international normalized ratio (INR), and aPTT patient:control (P:C) ratio results from Cornell Comparative Coagulation Laboratory.

Assay	Day −3	Day −2	Day −1	Day 0	MB	24 h	48 h	3 d	4 d	RI	Ctrl
Horse 2											
PT (s)	13.2	12.5	12.8	13.4	13.0	14.9	17.9†	18.8†	15.2	11–15	13.8
PT-INR	1.0	0.9	1.0	1.0	1.0	1.1	1.4	1.5*	1.2		
aPTT (s)	42.5	46.0	43.0	45.5	44.3	57.4	73.9*†	70.6*†	52.1	45–61	44.5
aPTT P:C	0.95	1.0	0.97	1.0	0.98	1.3	1.7*	1.6*	1.2		
Horse 3											
PT (s)	13.4	13.5	13.0	13.1	13.3	14.0	13.2	13.9	13.1	11–15	13.8
PT-INR	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.1	1.0		
aPTT (s)	47.0	49.3	46.8	46.0	47.3	53.0	50.7	55.4	49.4	45–61	44.5
aPTT P:C	1.1	1.1	1.1	1.0	1.1	1.2	1.1	1.2	1.1		

Ctrl = same-day control equine plasma; MB = mean baseline (days −3, –2, –1, 0); 24 h, 48 h, 3 d, and 4 d refer to post-administration intervals.
* Indicates values ≥1.5× MB.
† Indicates values outside of RI.

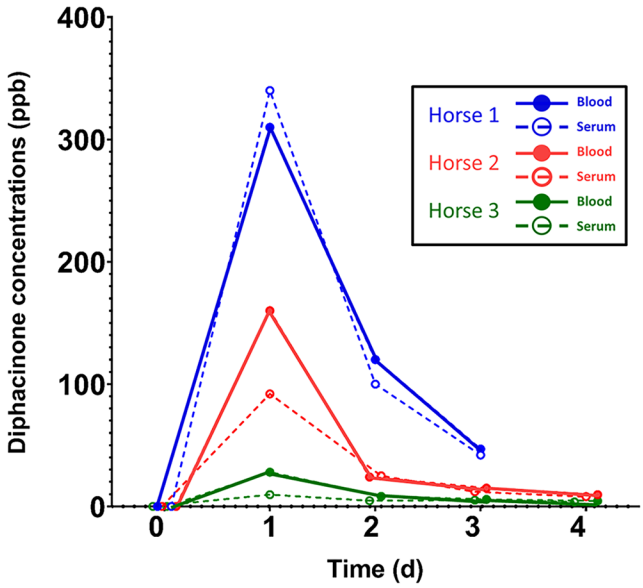

Figure 1. Whole blood and serum diphacinone concentrations over time. 0 = immediately prior to administration.

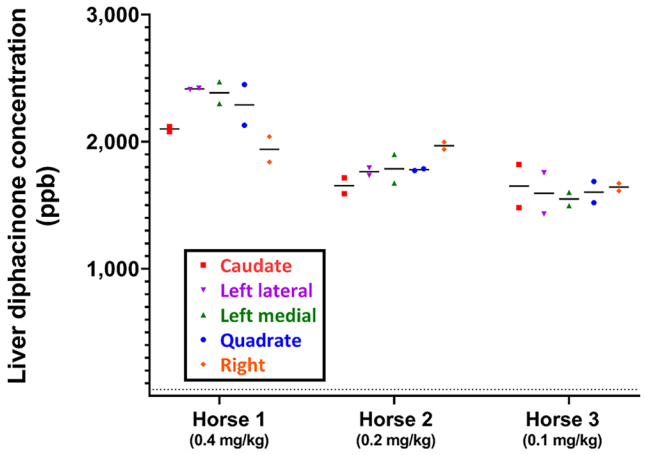

Figure 2. Postmortem liver diphacinone concentrations measured in each lobe. Concentrations are reported in ppb wet weight. Symbols denote liver lobes. Lines between symbols are means of replicates. Dotted line = minimum level of quantification (50 ppb).

target anticoagulant intensity.[8] In general, PT-INR target ranges are 2.0–3.0 for venous thrombotic syndromes, and 2.0–3.5 for valve replacement. Values >4.0–4.5 risk bleeding complications.

We used prolongation of both PT and aPTT as a proxy by which to detect combined deficiency of vitamin K–dependent clotting factors. Horse 2 (0.2 mg/kg dosage) developed prolonged aPTT and PT, whereas horse 3 (0.1 mg/kg dosage) did not. Therefore, we estimate a MTD of ~0.2 mg/kg diphacinone as a single oral dosage in horses. As in earlier equine studies, aPTT prolongation preceded PT prolongation.[3] Both aPTT and PT are prolonged with deficiencies of FII and FX. Specific deficiency of FVII causes prolongation of PT only; specific deficiency of FIX results in prolongation of aPTT and not PT. It has been suggested that, in horses, FIX has the shortest half-life of the vitamin K–dependent coagulation factors, in contrast to the relatively short half-life of FVII reported in studies of vitamin K antagonists in humans, rats, and dogs.[3,7,12] It is also possible that differences in PT reagent sensitivity to detect factor deficiencies may explain these discrepancies in test results among species. An alternative clotting time test, proteins induced by vitamin K absence (PIVKA), has been used in dogs as a more sensitive screening test to detect early vitamin K deficiency states after AR exposure.[9] In future studies, evaluation of the PIVKA test, or increased optimization of the PT assay, could be used to further explore coagulopathy induced by vitamin K antagonists in horses.

Table 3. Liver diphacinone concentrations by horse by dosage and liver lobe (2 technical replicates per lobe).

Liver lobe	Horse 1 (0.4 mg/kg)		Horse 2 (0.2 mg/kg)		Horse 3 (0.1 mg/kg)		Marginal mean	95% CI
Caudate	2,120	2,080	1,590	1,720	1,820	1,480	1,800	1,690–1,920
Left lateral	2,410	2,420	1,740	1,790	1,760	1,430	1,930	1,800–2,040
Left medial	2,300	2,470	1,680	1,900	1,500	1,600	1,900	1,790–2,030
Quadrate	2,450	2,130	1,790	1,770	1,520	1,690	1,890	1,780–2,010
Right	1,840	2,040	1,940	2,000	1,610	1,670	1,850	1,740–1,970
Median	2,290		1,780		1,600			

All concentrations reported in ppb wet weight. Marginal mean and 95% CI reported for each lobe. Median diphacinone concentrations reported for individual horses.

Limitations of our study include the small number of horses and the single-dosage protocol used. Many factors, including repeated exposures, can exacerbate the effects of AR poisoning in other species; this is likely also the case in horses. Coagulopathy resulting from combined deficiency of vitamin K–dependent clotting factors is inferred from the aPTT and PT prolongation in horse 2. Prolongation of aPTT occurred at ~48 h, consistent with the pharmacokinetics of AR compounds in other studies in horses. The transient nature of the prolongation is expected with a single-dose administration of a short-acting AR, given that dietary vitamin K replenishes endogenous stores. Neither clinically evident coagulopathy nor postmortem hemorrhage was observed in the horses with prolonged clotting times; however, if the horses had sustained even minor trauma, abnormal coagulation could have resulted in excessive bleeding. Therefore, we consider prolongation of both PT and aPTT as potentially significant indicators of coagulopathy. No such prolongation occurred in horse 3 (0.1 mg/kg), suggesting that 0.2 mg/kg is likely to be near the MTD. Our study provides a useful starting point for additional work.

We measured diphacinone concentrations in serum and whole blood to assess whether one matrix was preferable for confirmation of exposure in suspected poisoning cases. We found that both matrices were suitable; diphacinone was quantifiable from 24 h to 4 d post-exposure in either matrix, even in the horse administered the lowest dosage (0.1 mg/kg). Generally, whole blood concentrations were higher than serum concentrations, but this effect was not significant or consistent.

Although liver diphacinone distribution was uniform, individual measurements overlapped between horse 1 (1,840–2,470 ppb ww) and horse 2 (1,590–2,000 ppb ww) and between horse 2 and horse 3 (1,430–1,820 ppb ww). The degree of overlap between concentrations measured in horse 2 and horse 3 was especially surprising. Horse 2 received twice the dosage that horse 3 received, and the intervals between administration and euthanasia were nearly identical. Comparisons between horse 1 and horses 2 and 3 are more limited because of expected differences in bioavailability between the poorly soluble technical-grade diphacinone powder and the liquid diphacinone sodium salt. Also, the study endpoint was 4 d post-administration for horses 2 and 3 versus 3 d for horse 1. Liver diphacinone concentrations might have overlapped more if the intervals between administration and euthanasia had been similar in all 3 horses. We were surprised at the high concentrations in the livers of all 3 horses, including the horse with no clotting time prolongation. In contrast, much lower AR concentrations were associated with fatal hemorrhages in exercising horses at California racetracks.[2,4] In that report, diphacinone was detected in the liver of one horse by LC-MS/MS but was below the limit of quantitation of 0.25 ppm (250 ppb) and was designated "trace." Exercise, in addition to trauma, disease, and other factors, may have potentiated the effect of AR compounds.

Our pilot study represents an important first step in better interpreting results of diphacinone analysis in suspected equine poisoning cases. Follow-up studies detailing diphacinone clearance from serum or whole blood and liver could provide additional clarity. However, based on the similar diphacinone concentrations found in our study at both toxic and sub-toxic dosages, interpretation is likely to remain heavily reliant on the animal's clinical history, physical examination findings, and coagulation assessments.

Acknowledgments

We thank the staff at the Rood and Riddle Equine Hospital Laboratory and the Comparative Coagulation Laboratory for their technical assistance, and Dr. Marjory Brooks for helpful manuscript review and comments.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

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