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Detection and residence time of bisphosphonates in bone of horses

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Abstract. Bisphosphonates are potent anti-resorptive agents that have the potential to adversely affect bone healing in equine athletes, and normal bone adaption in young racehorses. A concern exists that bisphosphonate inhibition of normal bone metabolism could lead to increased bone fractures during high-intensity exercise. We found only a single report describing concentrations of tiludronate in the bone of horses, and no studies describing clodronate. Knowledge of the residence time in bone could allow for a better understanding of the long-term effects of these compounds. Our objectives were to develop a method for detection of bisphosphonates in bone and add to the limited information available regarding the disposition of these drugs in the bone of horses. Two horses received clodronate and 2 tiludronate disodium. Postmortem collection of bones and teeth occurred either 4 or 30 d post drug administration. Additionally, postmortem blood, synovial fluid, aqueous humor, and bone samples from racehorses with various histories of bisphosphonate administration were collected, and concentrations determined using the developed LC-MS/MS method. Bisphosphonates were detected in bones and teeth tested at 4 and 30 d. In a postmortem sample, clodronate was detected in bone from a horse with reported administration 18 mo prior; clodronate was not detected in other sample types collected from this horse. Bisphosphonates reside in bone for extended periods of time, which could lead to potential long-term effects, increasing the potential for bone fractures in young and/or athletic horses.

Keywords: bisphosphonates; bone; clodronate; horses; tiludronate.

Bisphosphonates are potent anti-resorptive agents that have been used for several years in human medicine to increase bone density and decrease fracture risk in individuals with osteoporosis.[1,7,14,16] In 2014, the U.S. Food and Drug Administration (FDA) approved 2 non-nitrogenous bisphosphonate products for use in veterinary medicine (clodronate and tiludronate disodium), labeled for the treatment of navicular syndrome in horses >4 y old.

Despite the recommendation of the FDA Center for Veterinary Medicine against the use of bisphosphonates in horses <4 y old (clodronate sodium; Osphos package insert, Dechra),[10] anecdotal reports exist of its extra-label use in this age group. To our knowledge, there are no published reports describing the pharmacologic effects of bisphosphonates on the skeleton of young horses. However, because this class of drugs is known to inhibit osteoclasts, and osteoclasts play an important role in bone development,[9] it follows that administration of bisphosphonates may have detrimental effects on normal bone adaption to race training, racing, or other high-intensity equine athletic activities. Of additional concern are the effects of bisphosphonates on bone healing, especially for horses participating in high-impact weight-bearing exercise such as horseracing. Microfractures are common in young racehorses,[15] and osteoclasts play a key role in repair. This has led to speculation that the use of bisphosphonates in young racehorses in training may interfere with the repair process, with microfractures progressing to catastrophic fractures.[15] In young human athletes, there have been reports of adverse effects of bisphosphonate administration on stress fracture healing.[13]

Information about concentration of bisphosphonates in horses is very scant. To our knowledge, there is only a single published report describing concentrations of tiludronate in the bone of horses,[4] and no studies describing clodronate levels. In humans and rats, it has been well established that, although bisphosphonates have a short residence (and detection) time in blood, they can remain in bone for months to years.[8] Given that bisphosphonates are only liberated from the bone in which they have deposited as the bone is resorbed, the half-life is dependent on the rate of turnover within that bone. It is also important to note that even after the drug is

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released from the bone, it can recirculate locally and systemically and reattach to bone surfaces, further prolonging the pharmacologic, specifically the anti-resorptive, effects.[8] The long residence time in human bone has led many physicians to recommend a drug "holiday" for bisphosphonates.[8] This "holiday" is a prolonged period of time whereby bisphosphonate administration is discontinued in an attempt to reduce the potential for adverse effects, such as atypical femoral fractures.[8] As has been described for humans, this could equate to an extended pharmacologic effect that could in turn result in disastrous consequences in young and/or athletic horses. Understanding the relationship between administration and the residence time of bisphosphonates in bone could allow for a better understanding of the potential long-term effects of these compounds and ultimately more judicious use of these compounds in horses.

We hypothesized that there is a similar prolonged residence time of bisphosphonates in the bones of horses, even though concentrations may be below detection limits in blood and urine. To that end, our primary objective was to develop a sensitive analytical assay that could be used to detect bisphosphonates in bones of horses collected postmortem as well as to continue to characterize the disposition of bisphosphonates in equine bone.

Materials and methods

Study design, horses, and drug administration

Our study was conducted in 2 phases. In phase 1, horses were administered a bisphosphonate, samples collected, and a sensitive analytical method developed. In phase 2, the method developed in phase 1 was used to test samples from horses that were examined as part of a postmortem program.

In phase 1, 4 healthy University of Kentucky–owned horses were studied including a 5-y-old Thoroughbred stallion, a 6-y-old mixed-breed stallion, a 14-y-old mixed-breed mare, and an 11-y-old Kentucky Mountain gelding. All horses were scheduled for euthanasia for reasons unrelated to our study. Our study was approved by the Institutional Animal Care and Use Committee of the University of Kentucky (Lexington, KY, USA; IACUC 2015-2101). Two horses received a single administration of clodronate disodium (Osphos; Dechra) per label instructions. A total IM dose of 1.8 mg/kg was administered, divided over 3 sites. For tiludronate disodium (Tildren; Bimeda) administration, an IV dose of 1 mg/kg was administered over 90 min. Horses were maintained on pasture following drug administration until the time of euthanasia.

In phase 2, postmortem bone samples from 4 horses (euthanized as a result of catastrophic musculoskeletal injury), collected as part of the California Horse Racing Board postmortem program at the University of California, Davis, were tested for the presence of clodronate and tiludronate disodium. Horses tested included those with a known

(based on veterinary records; horse 4) and unknown history of bisphosphonate administration (horses 1–3).

Sample collection

Phase 1. On day 4 post administration, one horse in the clodronate and one horse in the tiludronate disodium group were euthanized with pentobarbital, and bone samples were collected using standard laboratory protocols. The remaining 2 horses (1 clodronate-treated and 1 tiludronate-treated) were euthanized on day 30 post administration, and samples were collected as for day 4 horses. Samples collected included the left and right radius (day 4 only), the right third metacarpal bone (MC3), the right tuber coxae, and the right and left molars (day 30 only). Following collection, all bone and tooth samples were frozen at −20°C until processed for determination of drug concentrations by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Phase 2. All samples were collected in accordance with standard postmortem procedures as established by the California Horse Racing Board's postmortem program at the California Animal Health and Food Safety laboratory of the University of California, Davis. A bone sample (radius) and, when possible, additional biologic matrices, including blood, urine, synovial fluid, and aqueous humor, were collected and stored at −20°C until processed for determination of drug concentrations by LC-MS/MS.

Sample preparation, extraction, and concentration determination. Sample preparation, extraction, and concentration determination were the same for samples collected in both phases of this study and based on methods described previously.[6,11]

Serum, urine, synovial fluid, and aqueous humor sample preparation

Prior to analysis, 200 μL of serum was diluted with 100 μL of water containing 20 μg/mL of the internal standard etidronate (MilliporeSigma) or trimethyl orthoacetate (MilliporeSigma), 100 μL of 0.2 M HCl, and 2 mL of water. The samples were subjected to solid-phase extraction using Oasis WAX 60-mg columns (Waters). In brief, the columns were washed with 2 mL of methanol, and then 2 mL of water previously adjusted to pH 4 with formic acid. The samples were applied to columns, rinsed with 2 mL of pH 4 water again, rinsed with 2 mL of methanol, and eluted with 2 mL of 10% ammonium hydroxide in methanol. The samples were dried under nitrogen and re-dissolved with 150 μL of acetic acid and 500 μL of trimethyl orthoacetate before heating at 100°C for 30 min. After cooling to room temperature, 300 μL of 90% formic acid (aq.), 500 μL of water, and 3 mL of methyl tert-butyl ether were added, and the samples were mixed by

rotation (Glas-Col) for 20 min at 40 rpm. After rotation, samples were centrifuged at 3,300 rpm (2,260 × g) for 5 min at 4°C. The top organic layer was transferred to a glass tube, dried under nitrogen, and dissolved in 120 μL of 5% acetonitrile (ACN; Burdick and Jackson) in water, with 0.2% formic acid. The injection volume was 20 μL into the LC-MS/MS system (Thermo Scientific).

Bone and tooth preparation

Portions of bone and teeth, ~1 cm in length, were homogenized to a fine powder (Bead Ruptor 96; Omni International) and ~300 mg weighed and placed into tubes. A solution containing a 1:1 mixture of water:concentrated HCl (37%) and the internal standard were added, and the samples incubated overnight (20 h) at 40°C with constant shaking. After cooling to room temperature, the samples were filtered through 0.45-μm polytetrafluoroethylene filter frits. The samples were subjected to solid-phase extraction, and derivatization was performed as described above for serum. The calibration curve for bone and tooth samples was prepared by spiking control bone with clodronate and tiludronate.

Determination of drug concentrations

Clodronate and tiludronate working solutions were prepared by dilution of the stock reference solutions with 50% methanol in water to concentrations of 0.01, 0.1, 1, 10, and 100 μg/mL. Two stock solutions were prepared, one for calibrators and one for quality control (QC) samples. Serum calibrators were prepared by dilution of the working standard solutions with drug-free serum to concentrations of 2.5–1,000 ng/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, QC samples were included with each sample set as an additional check of accuracy. Serum and urine QC samples were prepared by fortifying drug-free matrix with analyte at 3 concentrations within the standard curve. For bone, QC samples were prepared by fortifying drug-free bone samples with analyte at one concentration within the standard curve. The concentrations of clodronate and tiludronate were measured in all matrices by LC-MS/MS (+). Quantitative analysis was performed on a TSQ Vantage triple-quadrupole mass spectrometer (Thermo Scientific) coupled with a turbulent-flow chromatography system (TFC TLX2; Thermo Scientific), with LC-10ADvp liquid chromatography systems (Shimadzu), and operated in laminar flow mode. Spray voltage was set at 3,500 V, sheath gas and auxiliary gas were 45 and 15, respectively (arbitrary units), and the vaporizer temperature was 300°C. Product masses and collision energies were optimized by infusing the standards into the mass spectrometer. Chromatography employed an ACE 3 C18 (10 cm × 2.1 mm) column (Mac-Mod Analytical) and a linear gradient of ACN in water with 0.2% formic acid, at a flow rate of 0.35 mL/min. The initial ACN concentration was held at 5% for 0.5 min, ramped to 90% over 5 min, and held at that percentage for 0.16 min, before re-equilibrating for 3.8 min at initial conditions. The retention times for clodronate and tiludronate were 3.5 and 4.9 min, respectively.

Detection and quantification were conducted using selective reaction monitoring of initial precursor ion for clodronate, mass-to-charge ratio 301 (m/z), tiludronate 375 (m/z), and the internal standard 327 (m/z). The response for the product ions for clodronate 209, 127, and 207 (m/z), tiludronate 217 and 155 (m/z), and the internal standard 175 and 267 (m/z) were plotted, and peaks at the proper retention time were integrated using Quan Browser software (Thermo Scientific).

Accuracy was reported as percent nominal concentration, and precision was reported as percent relative SD.

Results

The response of the LC-MS/MS instrument was linear and gave correlation coefficients of 0.99 or better. For clodronate and tiludronate, accuracy and precision were within 15% of the nominal concentration. The technique was optimized to provide a limit of quantification (LOQ) of 2.5 and a limit of detection (LOD) of 1.0 ng/mL for serum, synovial fluid, and aqueous humor for both analytes. For urine, the LOQ and LOD for both analytes were 5 and 2.5 ng/mL, respectively. For bone, the LOQ was 25 ng/g for both clodronate and tiludronate. The recovery for clodronate and tiludronate was 30%.

In phase 1, clodronate was detected in all bone and tooth samples collected on days 4 and 30 from horses administered clodronate (Fig. 1). Similarly, tiludronate was detected in all samples tested on both days 4 and 30 post drug administration (Fig. 1). The concentrations of drug were highest in the tuber coxae for both bisphosphonates on day 30.

In phase 2, neither clodronate nor tiludronate were detected in postmortem serum, urine, synovial fluid, or aqueous humor samples. Clodronate (0.42 μg/g of tissue), but not tiludronate, was detected in the bone sample collected from the horse with a previous history of clodronate administration. Neither bisphosphonate was detected in bone samples from the other 3 horses.

Discussion

A sustained detection time for bisphosphonates in bone with a prolonged elimination half-life of months to years has been reported in humans.[8] Although we did not collect blood samples from horses receiving clodronate or tiludronate in phase 1 of our study, the prolonged detection time in bone samples (30 d) suggests prolonged half-life of the drugs in horses. We found the highest concentrations of both bisphosphonates in the tuber coxae on day 30 post administration. This is similar to results of a previous study in horses, wherein tiludronate concentrations were measured in biopsy
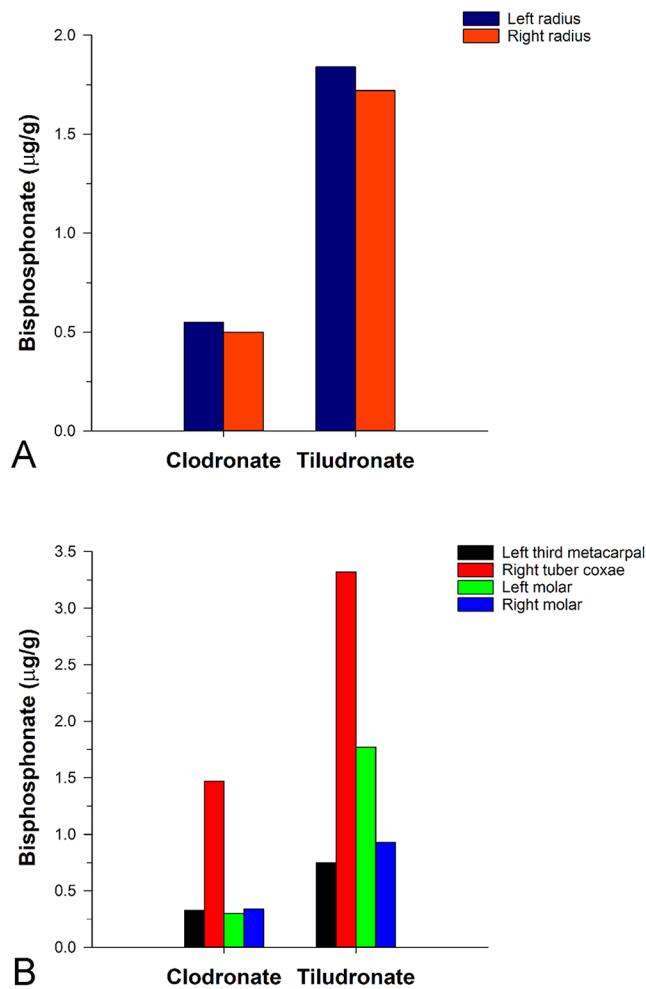
Figure 1. Clodronate and tiludronate concentrations in bone from horses ($n = 1$ per drug per time) administered a single administration of clodronate (1.8 mg/kg IM) or tiludronate (1 mg/kg IV over 90 min) either **A.** 4 d or **B.** 30 d before sample collection.

samples of the tuber coxae, the MC3, rib 13, and the cuboidal bone; with the exception of one horse, tiludronate concentrations were highest in the tuber coxae from 1 to 364 d post drug administration.[4] The authors attributed this distribution to the predominance of trabecular versus cortical bone in the tuber coxae, and the greater affinity of tiludronate for trabecular bone. The concentration of tiludronate that we found in the tuber coxae collected on day 30 post drug administration (3,250 μg/kg) was in close agreement with the concentration in the right tuber coxae reported on day 43 (3,257 μg/kg) in that study.[4]

We detected both clodronate and tiludronate in the right and left molars on day 30 post drug administration. Although to our knowledge there are no previous reports describing bisphosphonate concentrations in teeth, accumulation of drug in this matrix is not unexpected. Bisphosphonates are known to accumulate in the alveolar bone of the mandible, and with prolonged administration, osteonecrosis of the jaw

is a reported side effect.[2] The rate of turnover of mandibular alveolar trabecular bone is reportedly 6 times that of long bones, such as the femur,[5] presumably making mandibular molar areas more vulnerable to the effects of bisphosphonates. Although nitrogen-containing bisphosphonates have a greater tendency to accumulate in mandibular alveolar bone, there are reports of clodronate (a non-nitrogen–containing bisphosphonate) induced osteonecrosis of the jaw following prolonged administration.[3] Furthermore, there are reports of the therapeutic use of clodronate for dental disease.[12]

The prolonged detection time of bisphosphonates in bone in horses is further supported by the results observed in phase 2 of our study. Although clodronate concentrations in serum, synovial fluid, and aqueous humor samples collected postmortem from a horse (horse 4) with a known history of administration were below the LOD of our analytical assay, clodronate was detected in a bone sample collected from the same horse. Given that administration was reported to have occurred 18 mo prior to sample collection, our finding is in agreement with reports in humans describing prolonged detection time of bisphosphonates in bone, even when concentrations in blood are low or nondetectable.[8] The prolonged detection time of clodronate in the bone sample of the horse in phase 2 (horse 4) is also in agreement with a previous report[4] wherein tiludronate was detected in bone biopsy samples from the rib and tuber coxae collected from a horse 364 d post drug administration.

There are several limitations that require discussion in our study. Given that phase 1 was a terminal study, and phase 2 utilized horses suffering catastrophic injuries, the availability of animals and samples for study was limited. A larger number of study subjects would likely be more representative of the variability in disposition of bisphosphonates, especially with respect to distribution to tissues, such as bone. Drug disposition as well as the potential long-term pharmacologic effects of bisphosphonates may also vary based on age and exercise intensity. In phase 1, older non-exercised horses were studied, and it is likely that the extent of drug distribution and residence time in bone may have been different had a younger and/or exercising population of horses been studied. It is important to note when interpreting the results of our study that, although the bone and tooth samples collected were selected based on previous studies, they represent only a very small number of possible deposition sites for bisphosphonates.

Studies describing the pharmacologic effects of bisphosphonates on bone in horses are limited. However, because bisphosphonates are known to inhibit osteoclast activity, and osteoclasts play a role in bone development and remodeling, there is the potential for their administration to cause detrimental, even catastrophic, musculoskeletal injuries, especially in racehorses. Our sensitive analytical assay for the detection of bisphosphonates in bone provides a useful complementary tool for diagnosticians. Our study also provides evidence that, similar to reports in other species, this class of

drugs resides in bone for extended periods of time post administration to horses.

Declaration of conflicting interests

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

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