Received: 1 April 2020 | Accepted: 19 November 2020

DOI: 10.1111/evj.13395

GENERAL ARTICLE

Equine Veterinary Journal WILEY

Tiludronic acid can be detected in blood and urine samples from Thoroughbred racehorses over 3 years after last administration

Chris M. Riggs[1] | Sarah L. Thompson[1] | Yat-Ming So[2] | Jenny K.Y. Wong[2] | Terence S.M. Wan[2] | Paul Robinson[1] | Brian D. Stewart[3] | Emmie N.M. Ho[2]

[1]Veterinary Clinical Services, The Hong Kong Jockey Club, Sha Tin Racecourse, Hong Kong SAR, China

[2]Racing Laboratory, The Hong Kong Jockey Club, Sha Tin Racecourse, Hong Kong SAR, China

[3]Veterinary Regulation, Welfare and Biosecurity Policy, The Hong Kong Jockey Club, Sha Tin Racecourse, Hong Kong SAR, China

Correspondence
Chris M. Riggs, Veterinary Clinical Services, The Hong Kong Jockey Club, Sha Tin Racecourse, Sha Tin, N.T., Hong Kong SAR, China.
Email: christopher.m.riggs@hkjc.org.hk

Abstract

Background: Administration of bisphosphonates, including tiludronic acid, to Thoroughbred racehorses below 3 and a half years of age is prohibited in most racing jurisdictions.

Objectives: To determine if evidence of administration of tiludronic acid could be obtained from analysis of blood and urine samples beyond 40 days after administration.

Study design: Retrospective cohort.

Methods: Horses maintained in a highly controlled environment and treated with Tildren®[a] were selected from clinical records. Twenty-four horses were identified, 21 of which were still in race training. Blood and urine samples were collected and analysed for the presence of tiludronic acid using ultra-high-performance liquid chromatography–high-resolution mass spectrometry.

Results: Tiludronic acid was detected in samples from every horse, including two that had been given a therapeutic dose of the drug 3 years prior to sample collection. The estimated concentrations of tiludronic acid in the blood collected at least 2 years post-administration were consistently very low (less than 0.3 ng/mL). The estimated concentrations in urine were less consistent and were generally lower than those in blood, although higher levels were inconsistently detected in individual horses (up to about 16 ng/mL almost 1 year post-administration in 1 horse and about 3.7 ng/mL at almost 3 years post-administration in another).

Main limitations: The study was performed in horses that are older than the primary target group. A single sample was obtained from most horses and so we cannot comment on elimination profiles.

Conclusions: Evidence that a therapeutic dose of tiludronic acid has been administered to a horse can be obtained from detection of the drug in blood and urine samples over 3 years after it was administered.

KEYWORDS

bisphosphonates, doping control analysis, horse, liquid chromatography-mass spectrometry, tiludronic acid

1 | INTRODUCTION

Indiscriminate use of bisphosphonates in Thoroughbred racehorses is cause for concern. Bone modelling and remodelling are finely tuned physiological mechanisms that maintain skeletal health. These processes are responsible for adaptation of the skeleton to changes in exercise and for the ongoing repair and maintenance of bone tissue following injury.[1,2] Anything that impairs these homeostatic processes may lead to failure of the skeleton to adapt and/or increased fragility of bone tissue.[2] Bone resorption is the initial stage of both modelling and remodelling and so bisphosphonates, which inhibit resorption, have the potential to negatively impact these processes.[3] Furthermore, resorption of mineralised cartilage is a critical step during endochondral ossification, which is fundamental to the normal development of bones of the appendicular skeleton.[4]

Specific requirements for controlling the use of bisphosphonates in racehorses have recently been included in Articles 6D and 6E of the International Agreement on Breeding, Racing and Wagering (IABRW) by the International Federation of Horseracing Authorities (IFHA).[5] Specifically, no bisphosphonate may be administered to a racehorse in either of the following conditions: (i) under the age of 3 years and 6 months as determined by its recorded date of birth; and (ii) on the day of the race or on any of the 30 days before the day of the race in which the horse is declared to run. As such, there is a pressing need to develop a sensitive method that can effectively control the use of bisphosphonates in relation to preparation of bloodstock for sales and racing.

Bisphosphonates are drugs that are difficult to detect due to their intrinsic hydrophilic nature. However, methods have been successfully developed to extract bisphosphonates from either equine urine or plasma matrices.[6-9] These methods have been applied to analyses of post-administration samples collected from horses that had been administered with Tildren®[a] (tiludronic acid). Previously, the longest detection time of tiludronic acid was up to 40 days post-administration.[9] On the basis of a previously developed method,[7,8] herein we have enhanced this testing method to facilitate the detection of tiludronic acid at much lower concentrations. We hypothesised that by using this method, it would be possible to detect evidence of tiludronic acid for much longer than currently documented. We aimed to test this hypothesis by screening for the presence of residual tiludronic acid in blood and urine from Thoroughbred horses that were maintained in a highly controlled environment, and which had previously been treated with Tildren®[a].

2 | MATERIALS AND METHODS

2.1 | Samples

Samples for analysis were collected from Thoroughbred horses that were in race training or had been in race training at the Hong Kong Jockey Club (HKJC). The horses had previously been administered at least one dose of Tildren®[a] (tiludronic acid) as a prescribed treatment for a clinical condition. Tildren®[a] had been administered as a single intravenous 500 mg infusion in all but two horses, with those two horses each having received a lower dose (100 mg) by intravenous regional perfusion of the distal region of the left forelimb.

Medications and all clinical activities, including possession of syringes and needles, are strictly controlled at the HKJC. Under the HKJC's Rules of Racing, medications may only be administered by a veterinarian employed by the Club and infractions of this rule attract severe penalties. Medications and supplements are all processed through a central pharmacy in an Equine Hospital that is operated by the HKJC, which is subject to regular, independent audit. In addition, veterinarians are contractually required to document all clinical findings, prescriptions and administration of drugs in the Club's Veterinary Management Information System (VMIS) and, separately, in each Trainer's medication record book. Furthermore, there is a separate approval process for the use of Tildren®[a], which requires all Club veterinarians to obtain permission from the Head of Department before they may administer Tildren®[a] and the Pharmacy will not dispense this drug without the approval process having been completed. Besides testing on race days, the Club undertakes a thorough out-of-competition testing programme, which involves random sampling of all horses in training. These samples are subject to analysis for a wide range of prohibited substances and all findings are correlated with the medications recorded in the Trainer's medication record book and in the VMIS. Any discrepancies are investigated thoroughly. All horses in the study had been under the exclusive care of the Club for at least 6 months and all but 4 for over 1 year (Table S1). For all these reasons, there is a high degree of confidence that the records of when each horse had been medicated with Tildren®[a] were accurate and that horses could not have received additional doses other than those recorded.

A total of 149 Thoroughbred racehorses had been treated at the HKJC with Tildren®[a] since use of the drug was introduced. Of these, 50 were still present in Hong Kong with 23 in active race training (Figure 1). It was from this pool of 23 horses in active race training that 21 were sampled (the two remaining horses were not selected for practical reasons). One retired racehorse that remained in a trainer's stable as a "Lead Pony" was also selected and two retired racehorses were selected from a HKJC-owned equestrian centre. Horses in race training were aged between 3 and 7 years old (median 6 years), the Lead Pony was 5 years old, while the other two retired racehorses were 15 years old. The Lead Pony undertook regular track work and the two equestrian horses were used for pleasure riding. All horses were geldings. All but two horses had been treated with a single intravenous infusion of 500 mg Tildren®[a]. Two horses had been treated with a lower dose (100 mg) of Tildren®[a] by intravenous regional perfusion of the distal region of the left forelimb. Further details, including clinical reason for administration of Tildren®[a] to each case, date, dose and route of administration of Tildren®[a] and date of sampling for the current study are documented in Table S1.

Blood samples (two 8-mL Vacutainer gel tubes containing ethylenediaminetetraacetic acid [EDTA]) were collected from the left

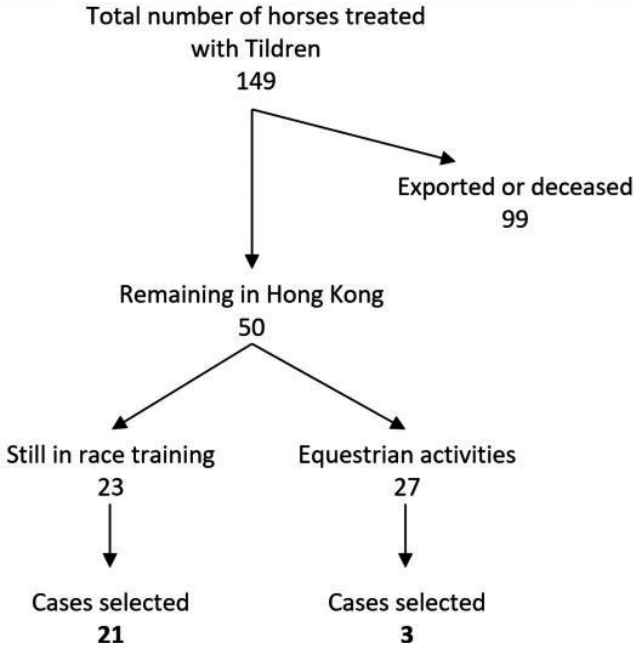


FIGURE 1 Selection of cases from all Thoroughbred racehorses that had previously been treated with Tildren®[a] at the HKJC

jugular vein. To evaluate whether tiludronic acid in plasma will bind to the gel in the blood tube, the analyses of two sets of three different negative equine plasma samples each spiked with 1 ng/mL of tiludronic acid and stored in either a blood tube with gel or a plastic tube without gel have been performed. Negative control samples of blank equine plasma were analysed in parallel. Analyses of the plasma in these blood tubes after storage for 1 and 2 days, respectively, both showed that the estimated concentrations of tiludronic acid determined in blood tubes containing a gel, for all three blood samples, were similar to those in plastic tubes without a gel, suggesting that the gel in the blood tubes did not affect the concentration of tiludronic acid determined (data not shown). Urine samples (at least 50 mL) were manually collected mid-stream during micturition. In all but five of the horses, blood and urine samples were collected within 12 hours of each other. The exceptions included 3 horses with urine samples collected 1, 3 and 30 days before blood collection, respectively, and the two horses with urine samples collected 28 days before blood (Table S1).

2.2 | Materials

Tiludronic acid (in the form of tiludronic acid disodium salt) and d_5-tiludronic acid were obtained from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Ammonia solution, potassium hydroxide and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). Sodium acetate and methanol were purchased from Honeywell Riedel-de Haën™ (Seelze, Germany). Hydrochloric acid and formic acid were purchased from Honeywell Fluka™ (Seelze, Germany). Benzyldimethylphenylammonium chloride (BDMPA) was obtained from ACROS (Fair Lawn, NJ, USA). Trimethylsilyldiazomethane (10% in hexane) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Oasis® HLB cartridge (60 mg, 3 mL) and Oasis® WAX cartridge (60 mg, 3 mL) were obtained from Waters Corporation (Massachusetts, USA). Deionised water was generated from an in-house water purification system (Milli-Q, Molsheim, France).

2.3 | Instrumentation

Ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS) analysis was performed on a Waters Acquity UPLC System (Waters Corporation) coupled to a Thermo Scientific™ Q Exactive™ mass spectrometer (Thermo Scientific™) with a heated electrospray ionisation (HESI-II) source. A Shimadzu LC-20AB binary pump (Shimadzu Corporation) was connected, post-column, through a T-joint for infusing benzyldimethylphenylammonium chloride (BDMPA) in deionised water (0.25 μg/mL) at a flow rate of 0.02 mL/min as the internal lock mass solution.

Chromatographic separation was performed on a reversed-phase Waters XBridge BEH C18 column (Waters Corporation; 15 cm × 2.1 mm ID; 3.5 μm particle size), with the column temperature set at 35°C. The mobile phase comprised of ammonium formate (5 mM, pH 3.0) as solvent A and 0.1% formic acid in acetonitrile (*v/v*) as solvent B. A linear gradient was run at a constant flow rate of 200 μL/min, with 98% solvent A and 2% solvent B at initial condition (t = 0 min), and then decreased linearly to 0% solvent A from t = 1 min to t = 10 min, and held for 5 min (until t = 15 min). The gradient was then returned to 98% solvent A and 2% solvent B from t = 15 min to t = 16 min, and stabilised until t = 20 min before the next injection. The injection volume was 5 μL.

The HESI-II source was set at 300°C and equipped with a high-flow metal needle insert. The sheath gas and auxiliary gas pressure were set to 50 and 10 arbitrary units respectively. The sweep gas pressure was set to 2 arbitrary units with curtain plate installed. The ion spray voltage was approximately + 3.0 kV, and the capillary temperature was set to 350°C. The S-Lens RF level was 40%. LC/HRMS product ion mass spectra were acquired using a mass resolution of 35,000 (full-width at half-maximum, FWHM at *m/z* 200) with an isolation window of 1 amu. The maximum injection time was 100 ms, and the automatic gain control (AGC™) was set at 2×10^5. Data processing was performed using the Thermo Finnigan Xcalibur (version 4.0) software with a mass tolerance window of ± 5 parts per million.

2.4 | Sample preparation for blood samples

The blood samples were centrifuged at 1,500 g for 10 min to separate out the plasma. The internal standard, d_5-tiludronic acid was added (equivalent to 25 ng/mL in the sample) to a portion of plasma (0.75 mL), which was then diluted with deionised water (2.5 mL). Trichloroacetic acid (50 μL) was added to the diluted plasma and the mixture was allowed to stand for 10 min at ambient temperature for

protein precipitation. The resulting mixture was centrifuged at 1,500 g for 10 min and the supernatant (3.3 mL) was collected for extraction.

2.5 | Sample preparation for urine samples

The urine samples were agitated to ensure thorough mixing and then 5-mL urine was aliquoted. From each aliquot, 1 mL of urine was pipetted out and mixed with 2 M HCl (0.5 mL). The acid-treated urine sample was centrifuged at 1,500 g for 10 min and the supernatant was collected. The internal standard, d_5-tiludronic acid (equivalent to 25 ng/mL in the sample) and deionised water (2.5 mL) were added to a portion of the supernatant obtained (0.75 mL), which was subsequently used for extraction.

2.6 | Extraction procedures

The urine and plasma samples were extracted according to the method developed by Wong et al.,[7,8] except that trimethylsilyldiazomethane (10% in hexane) was used instead of trimethylsilyldiazomethane (2 M in diethyl ether) and shaking the reaction mixture was included during the derivatisation process. Both measures have helped to improve the efficiency of derivatisation, thus improving the detectability of the present method (estimated limit of detection = 0.015 ng/mL for both urine and plasma, vide infra) compared with that of the previously reported method (estimated limit of detection = 10 ng/mL for both urine and plasma[7]). Sodium acetate solution (1 M, 0.4 mL) was added to the treated plasma or urine sample, and the mixture was adjusted to pH 4 and filtered through an Oasis® HLB cartridge (ACROS) that had been pre-conditioned with methanol (3 mL) and deionised water (3 mL). The filtrate was then loaded on an Oasis® WAX cartridge (Waters Corporation) that had been pre-conditioned with methanol (2 mL) and formic acid (2 mL, pH 4). The cartridge was washed with formic acid (2 mL, pH 4) and methanol (2 mL), and then eluted with ammonia solution (10% ammonia in methanol; 3.5 mL). A portion of the eluate (1 mL) was evaporated to dryness under nitrogen at 60°C, and then reconstituted with deionised water (25 μL), followed by methylation using a mixture of trimethylsilyldiazomethane (10% in hexane; 50 μL) and methanol (50 μL). The solution was incubated at ambient temperature for 1 hour with shaking at 250 rpm. A portion of the bottom layer (50 μL) of the solution was transferred to a Chrompack autosampler vial (La-Pha-Pack) containing methanol (50 μL) for LC/HRMS analysis.

2.7 | Estimation of the concentration of tiludronic acid in plasma and urine samples collected from horses administered Tildren®[a]

For plasma, two sets of calibrators were prepared by spiking blank equine plasma with tiludronic acid at, respectively: (i) 0, 0.1, 0.2, 0.4, 0.8 and 1.2 ng/mL; and (ii) 0, 1, 2, 4, 6 and 8 ng/mL. Two corresponding quality control equine plasma samples (spiked with 0.3 ng/mL and 2 ng/mL of tiludronic acid respectively) were also freshly prepared. All plasma samples were spiked with d_5-tiludronic acid (equivalent to 25 ng/mL in the sample) as the internal standard. The calibrators and the quality control plasma samples were analysed in parallel with the post-administration plasma samples. Both sets of calibrators gave straight lines using linear regression, with correlation coefficient greater than 0.99. The quality control plasma samples at 0.3 ng/mL and 2 ng/mL were analysed to be 0.29 ng/mL and 2.02 ng/mL, respectively, using the corresponding set of calibrators, and thus were well within ± 15% from the spiked concentrations.

For urine, two sets of calibrators were prepared by spiking blank equine urine with tiludronic acid at respectively: (i) 0, 0.1, 0.2, 0.4, 0.8 and 1.6 ng/mL; and (ii) 0, 5, 10, 20, 40 and 60 ng/mL. Two corresponding quality control equine urine samples (spiked with 0.4 ng/mL and 15 ng/mL of tiludronic acid respectively) were also freshly prepared. All urine samples were spiked with d_5-tiludronic acid (equivalent to 25 ng/mL in the sample) as the internal standard. The calibrators and the quality control urine samples were analysed in parallel with the post-administration urine samples. Both sets of calibrators gave straight lines using linear regression, with correlation coefficient greater than 0.99. The quality control urine samples at 0.4 ng/mL and 15 ng/mL were analysed to be 0.34 ng/mL and 14.83 ng/mL, respectively, using the corresponding set of calibrators, and thus were within ± 15% from the spiked concentrations.

For estimating the concentration of tiludronic acid, data acquisition was performed in the MS/HRMS mode, monitoring product ions *m/z* 156.98733 (derived from precursor ion *m/z* 375) for tiludronic acid and *m/z* 161.01244 (derived from precursor ion *m/z* 379) for d_5-tiludronic acid. The estimated limit of detection (LoD) and limit of quantification (LoQ) were determined to be 0.015 ng/mL and 0.05 ng/mL, respectively, for both plasma and urine matrices. The LoD and LoQ were determined by multiplying the standard deviation, obtained from analysing 10 blank plasma or urine samples spiked with a low concentration of tiludronic acid at 0.05 ng/mL, by 3 and 10 respectively [10].

3 | RESULTS

Paired urine and blood samples were collected from 24 horses that had been administered Tildren®[a] (tiludronic acid) as treatment for a range of clinical conditions ranging from approximately 1 month to just over 3 years prior to the date of sample collection (Table S1). Twenty-one of the horses were in active race training at the time the drug was administered, one was retired yet regularly undertook track work as a Lead Pony and two were retired to equestrian activities. Two horses had received one previous dose of Tildren®[a] and one three prior doses (Table S1).

Tiludronic acid was detected in urine and plasma samples collected from all 24 horses, including two horses that were administered a clinical dose of Tildren®[a] more than 3 years earlier (Table S2).

The mean estimated concentrations of tiludronic acid detected in the plasma of horses between 37 and 76 days after they had been administered 500 mg Tildren®[a] were about 2.6 ng/mL (±0.86). The mean estimated concentrations were about 0.40 ng/mL (±0.18) from horses between 286 and 335 days post-administration (Figure 2). The mean estimated concentration detected in plasma of five horses 2 years after being administered 500 mg Tildren®[a] was about 0.21 ng/mL (±0.03). The estimated concentrations of tiludronic acid in the plasma of the two horses treated by intravenous regional perfusion, each receiving a fifth of the normal systemic dose, were about 0.34 ng/mL at 55 days post-administration and about 0.04 ng/mL at 849 days.

The blood sample containing the lowest estimated concentration detected among the samples from all 24 horses (0.04 ng/mL) was subjected to confirmatory analysis by LC/HRMS. The relative abundances of the product ions of tiludronic acid in the plasma from this blood sample matched well with those of the corresponding positive control sample containing negative equine plasma that had been spiked with 0.03 ng/mL of tiludronic acid (Figure 3). Even though the estimated concentration of tiludronic acid in this sample was below the method LoQ but above the LoD, the presence of tiludronic acid was unequivocally confirmed by LC/HRMS analysis that has met all criteria stipulated in the *Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry* published by the Association of Official Racing Chemists.[11]

The excretion of tiludronic acid in urine appeared inconsistent between cases and while relatively high levels (compared with those in plasma) were detected in three cases, the estimated concentrations were generally lower than those present in plasma (Table S2). Surprisingly, high levels of tiludronic acid were detected in the urine of two horses 868 and 1,022 days post-administration; at about 2.5 and 3.7 ng/mL respectively (Figure 4). There was poor correlation between the estimated concentrations of tiludronic acid detected in plasma with that in the concurrent urine samples, despite blood and urine samples being collected within 12 hours of each other in all but five cases (Figure 5).

4 | DISCUSSION

The principal finding from this study is that it is possible to identify that a therapeutic dose of Tildren®[a] has been administered to a horse over 3 years after the drug was administered. The active agent in Tildren®[a], tiludronic acid, could be detected in the blood and urine samples from all three horses included in the study that had been treated over 1,000 days previously. Furthermore, tiludronic acid could even be detected 849 days after a fifth of the normal

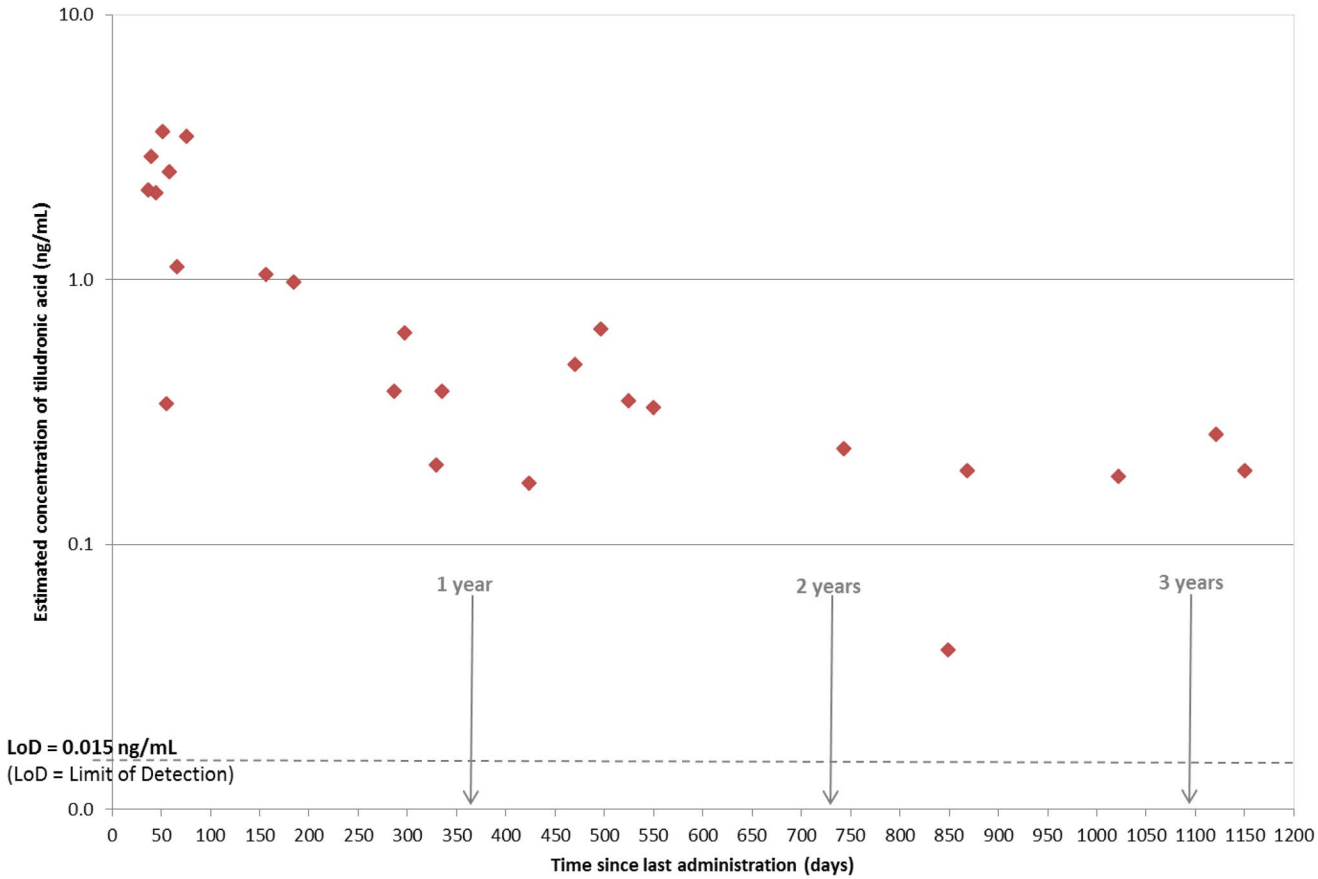


FIGURE 2 Estimated concentration of tiludronic acid in blood samples collected from 24 horses previously administered with Tildren®[a]. LoD = Limit of Detection

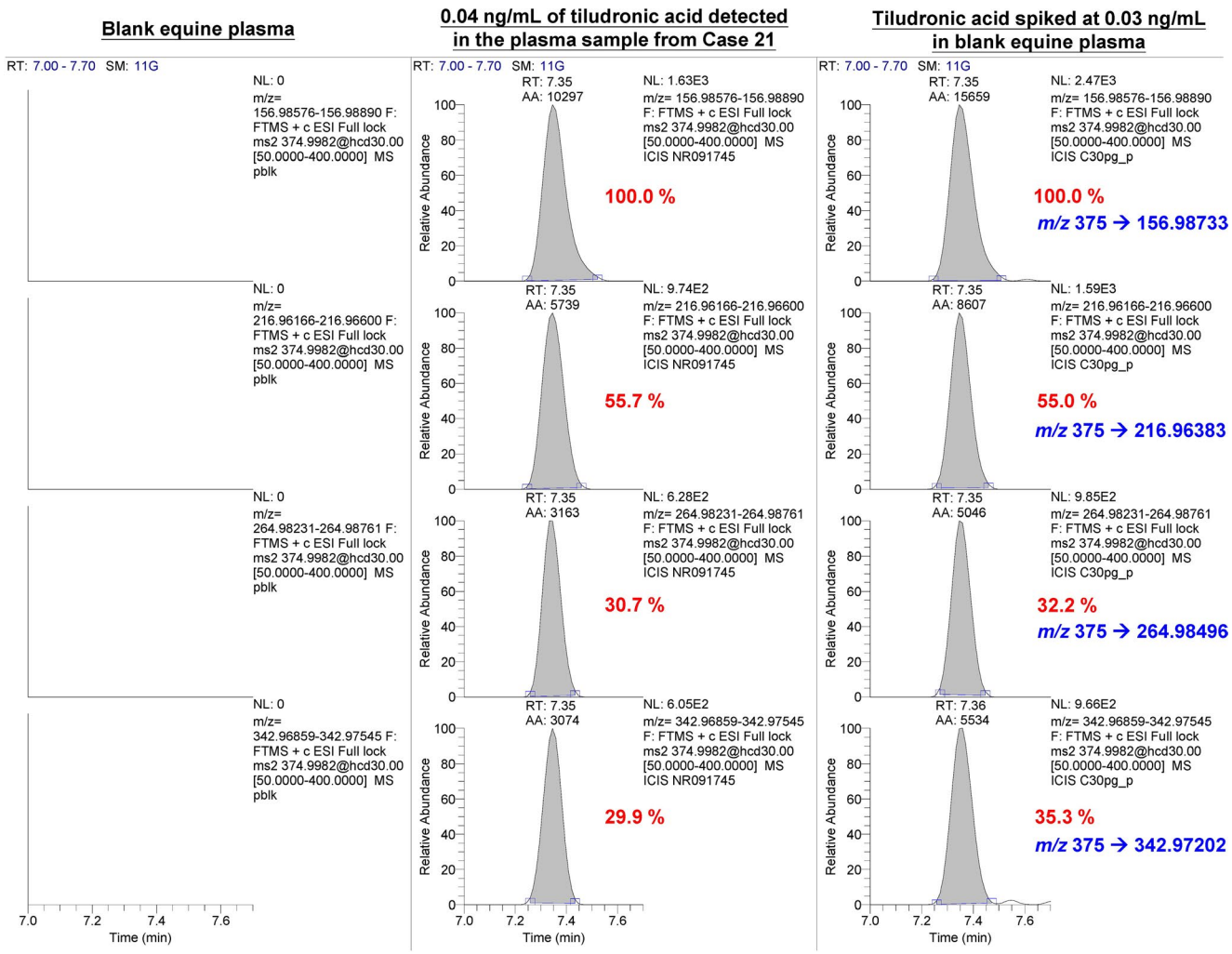


FIGURE 3 LC/HRMS confirmation of tiludronic acid in the plasma sample from Case 21

therapeutic dose had been administered to a horse by intravenous regional perfusion to the distal forelimb.

The longest interval previously reported between administration of a bisphosphonate to a horse and detection in blood was approximately 40 days.[9] The concentration of tiludronic acid in blood around 40 days after administration of 500 mg by intravenous infusion reported by Popot and co-workers (2018) was similar to that found here (about 2.5 ng/mL).[9]

This finding has important ramifications for the implementation of rules set by the IFHA, governing bodies of individual racing jurisdictions and for Bloodstock Sales Companies. For instance, in Great Britain, the British Horseracing Authority (BHA) dictates that any horse below the age of 3 years and 6 months that is administered a bisphosphonate drug will not be qualified to run under the BHA Rules of Racing at any point in its life.[12] Conditions of sales of most bloodstock auction companies globally now have specific statements relating to detection of bisphosphonates in horses sold through them, with the condition that the sale may be rendered void by the purchaser if bisphosphonates are detected in blood samples collected within a specified period following "fall of the hammer". Furthermore, sales companies in some jurisdictions are required by governing authorities to report any such finding for follow-up by regulators.

The consequences of detecting evidence of administration of a substance that is banned are very different from those of detecting evidence of prior administration of a permitted therapeutic agent. In the former, detection of any trace of the substance at any time represents a transgression. Conversely, most therapeutic substances are prohibited at the time of competition and yet evidence of their administration to an animal may be detected long after their therapeutic effect has worn off. In order to allow reasonable veterinary care this may require some leeway, which is generally managed by the use of screening or residue limits. This involves an estimate of the concentration of the drug or its specified metabolite detected in biological samples during a screening test. Whenever the estimated concentration in a particular sample exceeds the established screening or residue limit, confirmatory analysis will be conducted to prove the presence of the drug or metabolite in that sample. This approach is commonly used to manage,

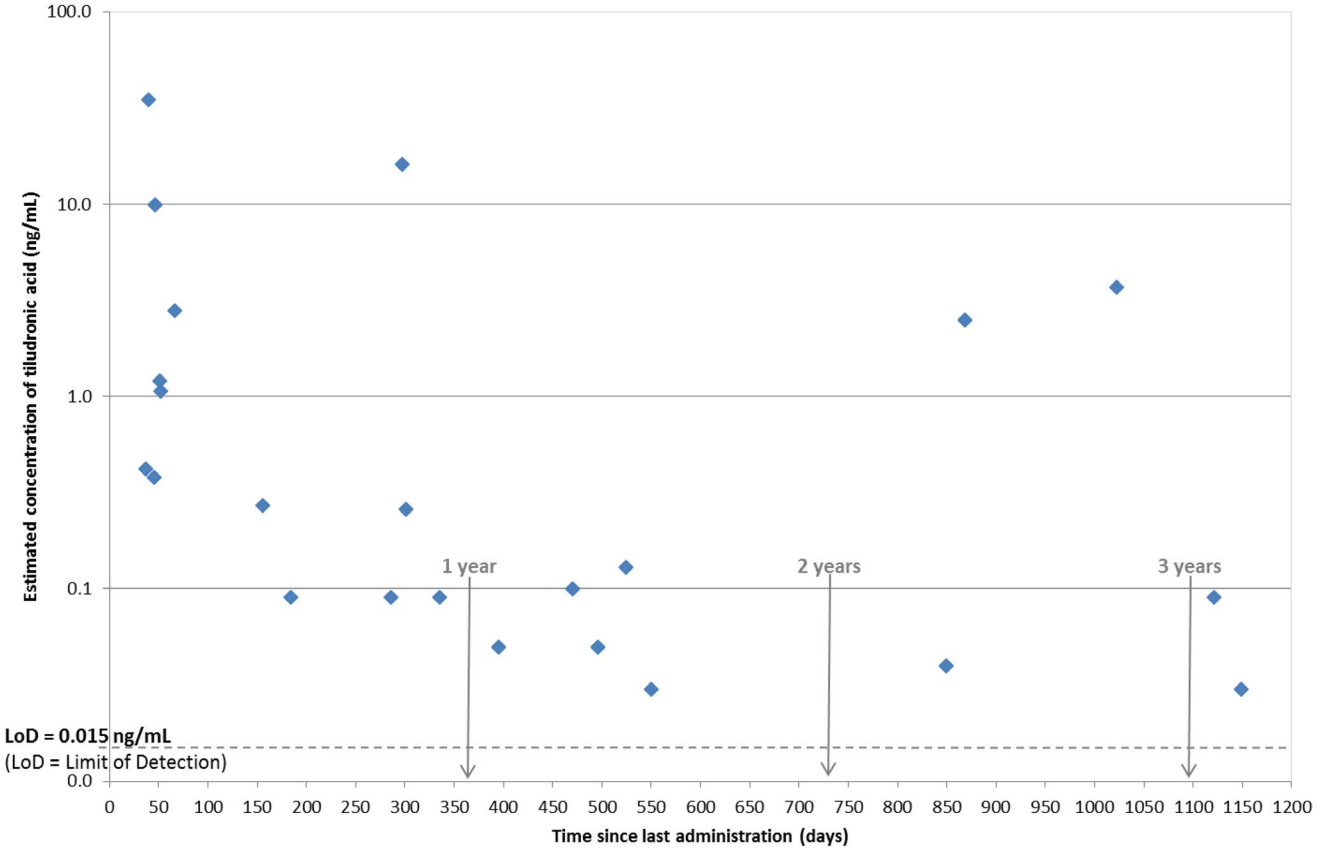
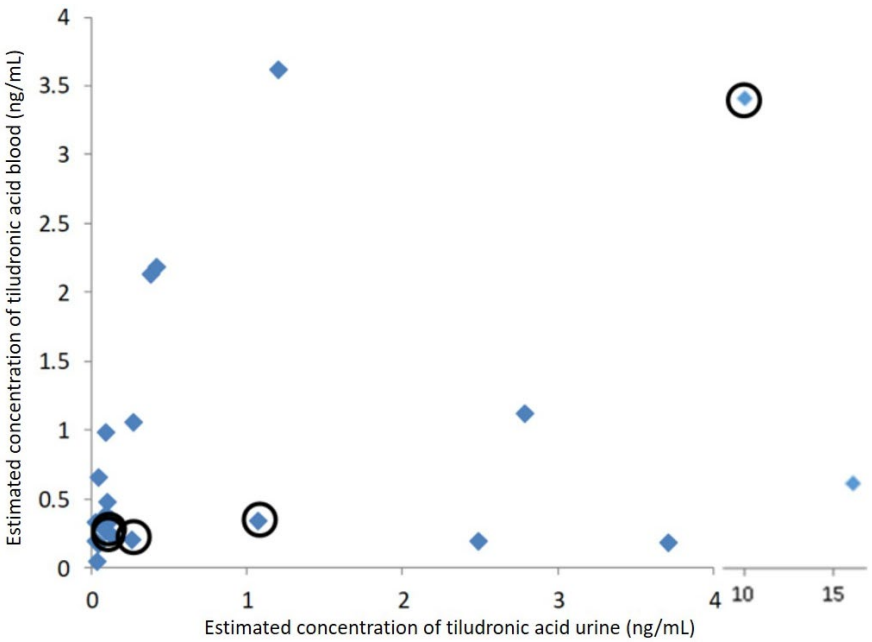


FIGURE 4 Estimated concentration of tiludronic acid in urine samples collected from 24 horses previously administered with Tildren®[a]. LoD = Limit of Detection

FIGURE 5 Relationship between the estimated concentrations of tiludronic acid detected in blood and urine. All but five samples (circled) were collected within 24 hours of each other



respectively, certain therapeutic substances and environmental substances. Clearly this approach may be necessary while bisphosphonates are still considered legitimate medicines in Thoroughbred racehorses for specific conditions in some racing jurisdictions.

The estimated concentrations of tiludronic acid detected in both urine and plasma were generally lower in horses sampled with increasing time post-administration. However, unexpectedly high levels of tiludronic acid were detected in urine samples from some horses many months after the drug had been administered. Both

urine and plasma results displayed scattered points with respect to the levels of tiludronic acid. This phenomenon may be due to individual horse-to-horse variations such as their health status and activity level (for non-resting horses) that would affect the elimination of tiludronic acid.[9] Bisphosphonates can remain bound to mineral in bone for years and pamidronate has been detected in urine from young people up to 8 years after cessation of treatment.[13] It is possible that consistent detection of low levels of tiludronic acid over 3 years after administration reflect the slow, steady elution of the compound from bone by background levels of bone resorptive activity. We speculate that periodic spikes in the concentration of tiludronic acid in urine may reflect times of significantly increased bone resorption. Osteoclastic bone resorption in bones of the distal limb of the horse is significantly more active during periods of relative inactivity,[14] and so release of tiludronic acid bound to mineral in bone may be affected by a horse's training schedule. Delguste and colleagues found that plasma carboxy-terminal cross-linking telopeptide of type I collagen, a reliable indicator of bone resorption, was significantly reduced in horses for only 3 days following administration of a single intravenous infusion of dose of Tildren®[a] that was the same as that used in the current study.[15] This suggests that the drug is only effective at inhibiting osteoclastic bone resorption at relatively high concentrations. Therefore, it is reasonable to speculate that resorptive activity in the horse skeleton is largely unaffected by the presence of this substance in the longer term, when concentrations will be very low. Consequently, change in osteoclastic activity in response to physiological drives could liberate bound tiludronic acid without the osteoclasts being unduly impacted by the liberated agent.

The lack of correlation between the estimated concentrations of tiludronic acid detected in concurrent blood and urine samples is interesting. We do not know if this reflects in part the variation in the specific gravity of urine that influence to a small extent quantification of the compound in this matrix, or more probably physiological considerations that may influence levels of excretion of tiludronic acid in the urine. In addition, there was frequently an interval of several hours between collection of blood and urine samples and so there may have been some diurnal effect on excretion that affected the data. This is potentially a topic for further investigation although it does alert to the fact that blood appears to be a more stable matrix for screening for presence of bisphosphonates.

All horses in Hong Kong are under the sole clinical care of vets employed by the HKJC. These clinicians are required to document accurately and promptly all clinical and therapies administered to every case. Furthermore, the Department of Veterinary Clinical Services has strictly regulated usage of Tildren®[a] (the only bisphosphonate used in horses in HKJC) since its use was first introduced, with an application form countersigned by the Head of Department required before the drug is dispensed by the Department Pharmacy. Consequently, we can be confident that the administration doses and dates recorded for each horse included in the study are accurate.

One case included in the study was less than 3.5-years-old at the time of administration. While this is below the timing threshold now approved by various racing jurisdictions, there were no such restrictions at the time of administration.

A limitation of the study is that samples were collected from horses that had been administered Tildren®[a] at an older age than one of the key groups of interest: horses younger than 3.5-years-old. It is conceivable that the pharmacokinetics may differ in younger horses that could influence the detection profiles. It is also possible that bisphosphonates may bind preferentially to calcified cartilage and release of drug bound to skeletal tissue may differ in younger animals. Also, we only obtained individual samples from most horses and so we cannot make any comment on elimination profiles.

5 | CONCLUSION

Evidence that horses had been administered a therapeutic dose of Tildren®[a] was obtained from detection of tiludronic acid in blood and urine samples in individual horses over 3 years after the drug had been administered. This has important ramifications for regulation of the use of this product in the field.

ETHICAL ANIMAL RESEARCH

This study was approved by the Hong Kong Jockey Club (HKJC) Animal Experimentation Ethics Committee.

ACKNOWLEDGEMENTS

We acknowledge and thank Hong Kong Jockey Club for providing us with the opportunity and resources to conduct this study. We are grateful to colleagues whose clinical material was used in the study. The authors also would like to thank Mr Ivan Cheung for his technical assistance in the analyses.

CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

C. Riggs, P. Robinson, B. Stewart and E. Ho contributed to the study design. All authors contributed to the study execution, and data analysis and interpretation. C. Riggs and E. Ho prepared the manuscript. All authors approved the final manuscript. C. Riggs and E. Ho have full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data.

FUNDING INFORMATION

There was no specific funding for this project.

INFORMED CONSENT

Owners' consent was obtained via rules of ownership of a horse in training at the HKJC.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/evj.13395.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Chris M. Riggs https://orcid.org/0000-0002-9332-4789
Yat-Ming So https://orcid.org/0000-0003-0015-781X
Jenny K.Y. Wong https://orcid.org/0000-0001-8245-6770
Terence S.M. Wan https://orcid.org/0000-0003-3779-2649
Emmie N.M. Ho https://orcid.org/0000-0001-8366-2066

REFERENCES

1. Ehrlich PJ, Lanyon LE. Mechanical strain and bone cell function: a review. Osteoporos Int. 2002;13:688–700.
2. García-Aznar JM, Rueberg T, Doblare M. A bone remodelling model coupling microdamage growth and repair by 3D BMU-activity. Biomechan Model Mechanobiol. 2005;4:147–67.
3. Bajaj D, Geissler JR, Allen MR, Burr DB, Fritton JC. The resistance of cortical bone tissue to failure under cyclic loading is reduced with alendronate. Bone. 2014;64:57–64.
4. Ballock RT, O'Keefe RJ. The biology of the growth plate. J Bone Joint Surg Am. 2003;85:715–26.
5. International Federation of Horseracing Authorities (IFHA). Article 6 in the International Agreement on Breeding, Racing and Wagering (IABRW). https://www.ifhaonline.org/default.asp?section=IABRW&area=3. Accessed June 4, 2020
6. Popot MA, Garcia P, Hubert C, Bolopion A, Bailly-Chouriberry L, Bonnaire Y, et al. HPLC/ESI-MSn method for non-amino bisphosphonates:application to the detection of tiludronate in equine plasma. J Chromatogr B. 2014;958:108–16.
7. Wong ASY, Ho ENM, Wan TSM, Lam KKH, Stewart BD. Liquid chromatography-mass spectrometry analysis of five bisphosphonates in equine urine and plasma. J Chromatogr B. 2015;998:1–7.
8. Wong ASY, Leung GNW, Ho ENM, Wan TSM, Lam KKH, Stewart BD. (2020) Improved liquid chromatography-mass spectrometry analysis of bisphosphonates in equine plasma. Proc 22th Int Conf of Racing Analysts and Veterinarians, Dubai, 3–10 March 2018; in press.
9. Popot MA, Jacobs M, Garcia P, Loup B, Guyonnet J, Toutain PL, et al. Pharmacokinetics of tiludronate in horses: a field population study. Equine Vet J. 2018;50:488–92.
10. Thomsen V, Schatzlein D, Mercuro D. Limits of Detection in Spectroscopy. Spectroscopy. 2003;18:112–4.
11. Association of the Official Racing Chemists, Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry. http://www.aorc-online.org/documents/aorcms-criteria-modified-23-aug-16/. Accessed June 4, 2020
12. BHA Notice: Bisphosphonates (10 August 2017). https://www.britishhorseracing.com/wp-content/uploads/2018/05/BHA-Notice-Bisphosphonates.docx.pdf. Accessed on June 4, 2020
13. Papapoulos SE, Cremers SCLM. Prolonged Bisphosphonate Release after Treatment in Children. N Engl J Med. 2007;356:1075–6.
14. Holmes JM, Mirams M, Mackie EJ, Whitton RC. Thoroughbred horses in race training have lower levels of subchondral bone remodelling in highly loaded regions of the distal metacarpus compared to horses resting from training. Vet J. 2014;202:443–7.
15. Delguste C, Amory H, Guyonnet J, Thibaud D, Garnero P, Detilleux J, et al. Comparative pharmacokinetics of two intravenous administration regimens of tiludronate in healthy adult horses and effects on the bone resorption marker CTX-1. J Vet Pharmacol Ther. 2008;31:108–16.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Riggs CM, Thompson SL, So Y-M, et al. Tiludronic acid can be detected in blood and urine samples from Thoroughbred racehorses over 3 years after last administration. *Equine Vet J*. 2021;53:1287–1295. https://doi.org/10.1111/evj.13395