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Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: A field study

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Summary

Background: Methylprednisolone (MP) acetate is a commonly used corticosteroid for suppression of inflammation in synovial structures in horses. Its use is often regulated in equine sports by plasma MP concentrations.
Objectives: To describe variability in MP plasma concentrations after MP acetate injection in different synovial structures and with co-administration with hyaluronic acid (HA).
Study design: Field study in actively racing horses in three disciplines (Thoroughbred, Standardbred and Quarter Horse).
Methods: Seventy-six horses (15 Thoroughbreds, 20 Standardbreds and 41 Quarter Horses) were included in the study. Injection of any synovial structure with a total body dose of 100 mg MP acetate was permitted, data were grouped according to the synovial structure injected and co-administration with HA. Plasma was collected before injection and at 6 days post-injection. Per cent censored data (below the limit of quantification) for each synovial structure were determined, and summary statistics generated by Robust Regression on Order. Differences between synovial structures and co-administration with HA were identified by ANOVA with Tukey's post hoc testing.
Results: The MP plasma concentration at 6 days for injection for the entire group (mean ± standard deviation [s.d.], pg/mL) was 96 ± 104. Metacarpophalangeal (MCP) plasma concentrations contained 86% censored data and could not be included in the statistical analysis. The carpal joints (CJO) group had a lower plasma MP concentration ($P<0.05$) than the distal tarsal joints (DTJ) or medial femorotibial (MFT), the no HA (NHA) group had a lower plasma MP concentration ($P<0.05$) than HA.
Main limitations: The synovial structures injected varied by racing discipline, so this study was unable to identify any differences between disciplines.
Conclusions: Practitioners should be aware that injection of DTJ, CS and MFT joints, and combining MP acetate with HA may prolong its clearance, and withdrawal times for competition in regulated equine sports.

Keywords: horse; methylprednisolone; joint injection; racehorse; threshold; plasma concentration

Introduction

Methylprednisolone acetate, pregna-1,4-diene-3,20-dione, 21-(acetyloxy)-11,17-dihydroxy-6-methyl-,(6α,11β), molecular weight 416.51 g/mol (MP acetate) (Depo-medrol®)[a] is an FDA-approved long-acting, slightly water-soluble corticosteroid prodrug for intrasynovial administration in horses where targeted therapy is recommended. The marginal water solubility of MP acetate delays its entry into joint fluid, accounting for its prolonged therapeutic action. Once dissolved in joint fluid the acetate prodrug moiety is catalysed by alkaline hydrolysis/esterase action, yielding the therapeutically active molecule, methylprednisolone.

Over 53% of racehorses [1] experience lameness during their racing careers, of which joint injury is one of the major causes. As many as 27% of Thoroughbred yearlings go through public auction with pre-existing arthritis [2], and this problem is not limited to Thoroughbreds, as a comparable 33% prevalence is seen in nonracing breeds [3]. Targeted joint therapy for these conditions using MP acetate has been a mainstay of equine veterinary care since the early 1960s. Use of this specific therapeutic medication is accompanied by restrictions in proximity to competition in performance horses with most international sports and racing jurisdictions providing withdrawal guidelines without published thresholds. The Canadian Para-Mutuel Agency (CPMA) recommends a 6-day withdrawal after 100-mg intra-articular (IA), or a 14-day withdrawal after 200 mg [4], whereas the Federation Equestre Internationale provides a detection time of 14 days after 100-mg IA and 28 days after 200-mg IA [5].

In the United States, many jurisdictions have adopted a regulatory threshold of 100 pg/mL MP in plasma or serum pursuant to a recommendation by the Racing Medication and Testing Consortium (RMTC), which is accompanied by a withdrawal recommendation of 21 days. The RMTC typically determines thresholds based on the application of a statistical method called the 95/95 tolerance [6], although it is not clear whether this statistical method was used in the case of MP. The RMTC references a pharmacokinetic study of 16 research horses [7], in which MP acetate injection was restricted to a single dose of 100 mg without concomitant medication in one antebrachiocarpal joint of Thoroughbreds, and these data were used to determine both MP plasma pharmacokinetics and support their threshold recommendation. The regulated population of racing horses that receive MP acetate may have injections into joints other than the antebrachiocarpal joint, and is comprised of horses other than Thoroughbreds. In the case of at least one therapeutic medication, glycopyrrolate, pharmacokinetic properties differ between Thoroughbreds and Standardbreds [8].

In order to provide guidance for horsemen and veterinarians operating under regulatory restrictions, including the effect of synovial structure being injected and co-administration of hyaluronic acid, this study was

performed. We sought to include horses of all three major racing disciplines, Thoroughbreds, Standardbreds and Quarter Horses under actual training conditions and to evaluate the effect of the widely used co-administration with hyaluronic acid (HA) in order to provide guidance to practitioners for practical therapeutic use of MP acetate. We chose 6 days post-MP acetate administration for collection of plasma samples because this time frame was likely to minimise the censored data points, or data below the limit of quantification (LOQ), based on the previous work [7].

Materials and methods

Study facilities and animals

Privately owned Thoroughbred, Standardbred and Quarter Horse racehorses in race training in the practice population of five of the authors (C.F., D.W., M.I., B.B. and S.A.) were used throughout. Horses were stabled on the racetrack or at training centres and were housed and trained according to standard procedures at racing facilities in Kentucky, Ohio and New Mexico. The feed, bedding and water sources were consistent with routine management at each facility. Training adhered to regimens consistent with the type of racing specific to the racing discipline. Informed consent was obtained for all horses enrolled. Inclusion criteria were a full clinical examination and a diagnosis requiring joint and/or synovial compartment therapy with 100-mg MP acetate total body dose. Treatment was based on the exam and diagnosis by the examining investigator, a signed owner consent form, long-term trusted relationships between the investigator and trainer to insure trainer compliance and active participation in racing or fast workouts in preparation for racing. Exclusion criteria were previous injections with MP acetate within 1 month, or any other medications within the 24 h prior to blood collection.

Experimental design

All racehorses in the five investigators' practices which fulfilled the inclusion criteria with none of the exclusion criteria were enrolled in the study. In order to replicate the usual clinical usage patterns of MP acetate, no restrictions were placed on co-administered medications. The synovial structure injected was recorded, without any restrictions on which structures could be injected. The injected structures were the medial femorotibial joint (MFT), distal intertarsal joint and tarsometatarsal joint (DTJ), distal interphalangeal (DIP), antebrachiocarpal and intercarpal (combined as CJO), metacarpophalangeal joints (MCP) and carpal sheath (CS). Blood was collected into lithium heparin tubes prior to synovial structure injection(s), and post-injection blood was collected in all cases 6 days (±2 h) after synovial structure injection. In five cases where preinjection samples were not collected, a complete review of the horse's medical record for the last month was performed to ensure that no prior injection with MP acetate had occurred. Blood was kept refrigerated at 4°C and shipped overnight to the New York Drug Testing and Research Laboratory for analysis.

Analytical methods

The analytical procedure followed was the ISO 17025/RMTC accredited quantitative analytical procedure for Methylprednisolone in place in the New York Drug Testing and Research Program. The reference standard for MP was purchased from Sigma Aldrich[b]. The analytical reference standard MP-d_2 used as internal standard was purchased from CDN Isotopes[c]. Stock solutions of MP and the internal standard were prepared at 1 mg/mL in methanol. Acetonitrile and methanol were purchased from EMD Millipore[d], and methyl-tert-butyl ether and ammonium formate were purchased from Fisher Scientific[e]. Deionised water was filtered onsite to the specification of 18.2Ω. All reagents were HPLC grade or better.

Methylprednisolone working solution was prepared by dilution of the 1 mg/mL stock solution with ethanol to the concentration of 25 pg/μL. Plasma calibrators were prepared by dilution of the working standard solution with drug-free plasma to concentrations of 50, 100 and 200 pg/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay.

Prior to analysis, 1 mL of plasma was aliquoted into new, labelled test tubes. To each tube, MP-d2 stock solution was added along with 5 mL of methyl-tert-butyl ether. The samples were mixed by rotation for 10 min, centrifuged at 2400 rpm for 5 min and the top ether layer removed and dried under nitrogen. Samples were dissolved in 100 μL of 1:1:1 acetonitrile:methanol:DI water and a 2.5-μL aliquot injected into the LC-MS/MS system, Agilent 6400 series triple quadrupole mass spectrometer[f] coupled with an HPLC chromatography system.

The concentration of MP was measured in plasma by LC-MS/MS using positive electrospray ionisation. The mass spectrometer was operated using electrospray combined with Agilent's Jet Stream Technology[f]. Chromatography employed a Zorbax SB-C18 column with specifications of 10 cm × 3.0 mm, 3.5 μm, column[e] and a linear gradient of acetonitrile (ACN) in water with a constant 5 mmol/L ammonium formate (pH 3.5) at a flow rate of 0.5 mL/min. The initial ACN concentration was held at 40% for 3.0 min, ramped to 95% over 1.0 min and held at that concentration for 1.0 min before re-equilibrating for 0.5 min at initial concentration.

Detection and quantification were conducted using selective reaction monitoring (SRM) of initial precursor ion for MP (mass-to-charge ratio 375.2 m/z) and the internal standard (361.2 m/z). The response for the product ions for MP (m/z 339, 321, 293 and 253) and the internal standard (m/z 161) was plotted and peaks at the proper retention time integrated using MassHunter software[f]. MassHunter software was used to generate calibration curves and quantitate MP in all samples by linear regression analysis.

The validation of the method employed for the analysis of MP contained a calibration curve performed encompassing 50, 100 and 200% of the threshold value for MP. The response was linear and gave correlation coefficients (R2) of 0.99 or better. Quality control samples replicates were performed (n = 7). The interday accuracy was 5.5% for 100 pg/mL MP. The intraday accuracy was 0.3% for 100 pg/mL MP. The interday precision was 2.5% for 100 pg/mL MP. The intraday precision was 13.9% for 100 pg/mL MP. The technique was optimised to provide a lower limit of quantification (LOQ) of 0.05 ng/mL. The limit of detection (LOD) was 0.025 ng/mL. This analytical method has been shown to exclude 20-dihydro-6-methylprednisolone, an isomeric metabolite of methylprednisolone in the horse and a possible confounding isomeric metabolite of methylprednisolone with respect to the unequivocal identification and confirmation of methylprednisolone in post-MP administration equine plasma samples [9].

Data analysis

The 6-day post-administration plasma MP concentrations were analysed for percentage of censored (below LOQ) data, effects of number of synovial structures injected, specific synovial structure injected and co-administration with HA using Robust Regression on Order [10] and General Linear Model statistical methods [11]. Data were first grouped as all horses, then subgrouped according to the synovial structure, number of synovial structures injected and concomitant administration of HA. Where multiple injections resulted in horses being categorised into more than one group, the data from the overlapping groups were compared with each individual group in order to determine if these data should be compared separately. The effect of HA on plasma clearance was also included as a separate analysis of co-administration with HA (HA) or no co-administration with HA (NHA).

Each dataset was first analysed for per cent censored data, then Normality tests (Shapiro-Wilk, Anderson-Darling, Lillefors and Jarque-Bera) were performed on uncensored (above LOQ) data in order to determine the most appropriate statistical analysis for threshold determination. Summary statistics for each data subgroup were obtained for all groups except CS data and MCP data using RROS [11] in R-programming language and bootstrap analyses with 100,000 resamplings (XLSTAT®, ADDinsoft 2016 https://www.xlstat.com/en/ as a Excel® for Mac 2011, Microsoft add-in). Bootstrap with resampling was performed to improve estimates of group summary statistics. Comparisons of the grouped Synovial structure data were then performed using ANOVA, with Tukey's post hoc testing. The use of concomitant HA and no HA groups was compared in a separate analysis using a *t* test. The effect of the number of joints injected was compared using joints as count data with a General Linear Model in R being cognisant of the effects of any overdispersion [11], and

homoscedasticity of variances tested by Bartlett and Brown-Forsythe tests [12]. Thresholds for each data set were determined using two different methods: (95/95) tolerance interval [6] and Gauss-Camp-Meidell (GCM) [13].

Results

Seventy-six horses met the inclusion criteria (15 Thoroughbreds, 20 Standardbreds and 41 Quarter Horses). Sixty-eight horses had preinjection plasma samples analysed, and all of these plasma samples were below the LOD of the analytical method for MP. Seven horses did not have pre-injection plasma samples analysed, but a review of the medical history for these horses showed no previous MP administration or exposure, and these horses were included in the analysis.

The MCP data set had 86% censored data, so no further analysis could be conducted [10]. In all cases where the DIP joint was injected, the MCP joint was also injected, so no separate conclusions or analyses could be done on this subgroup. There were 13 instances of horses categorised into both the MCP and CJO groups. Of these, 12 were censored. There was one instance of a horse categorised into the MFT and CJO group (censored), and one instance of a horse categorised into the MFT and DTJ groups (uncensored). All 15 were included in both groups for analysis. The per cent censored data for each group is shown in Table 1. Normality tests (Shapiro-Wilk, Anderson-Darling, Lillefors and Jarque-Bera) for each data set (All horses, CJO, CS, DTJ, MFT, HA and NHA) indicated that noncensored data (plasma concentrations above the LOQ) were normally distributed.

Where censored data in groups fell below 80% (All horses, CJO, CS, DTJ, MFT, HA and NHA groups), summary statistics for plasma concentrations arising from MP acetate intrasynovial injections were obtained as described above. Standard parametric summary statistics were used for CS data, as recommended by Helsel [10]. Box and whisker plots (mean, interquartile range and highest and lowest scores, with outliers indicated) for these data are shown in Figures 1 and 2, and differences indicated in the figures.

Breed/racing discipline differences were found among which specific synovial structures were commonly injected with MP. Insufficient horse numbers were present for each joint or synovial structure injected among the different breeds, and not every breed was represented in all injection groups (Table 2). Therefore, all breeds were combined, and the differences were analysed by the MP-treated synovial structure. No effect of the number of synovial structures injected on the MP plasma concentrations was found (Fig 3, $P = 0.8$) with lack of overdispersion. There were 13 instances where CJO and MCP were co-injected and one instance where DTJ and MFT were co-injected. In all cases, the resulting MP plasma concentrations were within the interquartile range of both groups, so the case was included in the analysis of both groups.

TABLE 1: The distribution of censored (plasma concentrations methylprednisolone below the limit of quantification) and uncensored (plasma concentrations methylprednisolone at or above the limit of quantification) among 6-day methylprednisolone plasma concentrations. Horses that were injected into multiple synovial structures are included in both groups and include 13 horses in both the metacarpophalangeal and carpal joints, one horse in both the medial femorotibial and carpal joints and one horse in both the medial femorotibial and distal tarsal joints

Injection site	Total	#Censored	%Censored	%Uncensored
All horses	76	46	61	39
Carpal joints	36	25	69	31
Carpal sheath	4	0	0	100
Distal tarsal joints	7	1	6	94
Metacarpophalangeal	34	29	85	15
Medial femorotibial	10	4	40	60
Hyaluronic acid	21	10	48	52
No hyaluronic acid	55	36	65	35

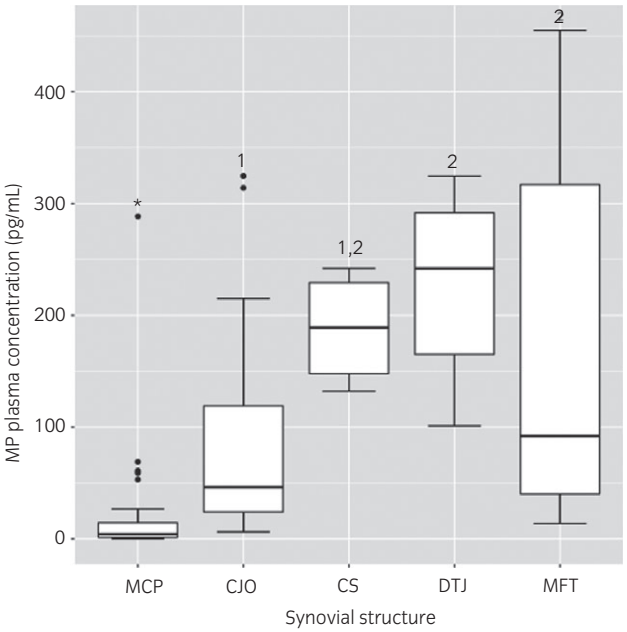

Fig 1: Box and whisker plots for plasma methylprednisolone concentrations 6 days post-injection by synovial structure. Concentrations that differ by structure are indicated by different letters. *Metacarpophalangeal data are shown for comparison but were not included in the analysis because the censored data (below the limit of quantification) exceeded 80%, precluding this group from analysis [10].

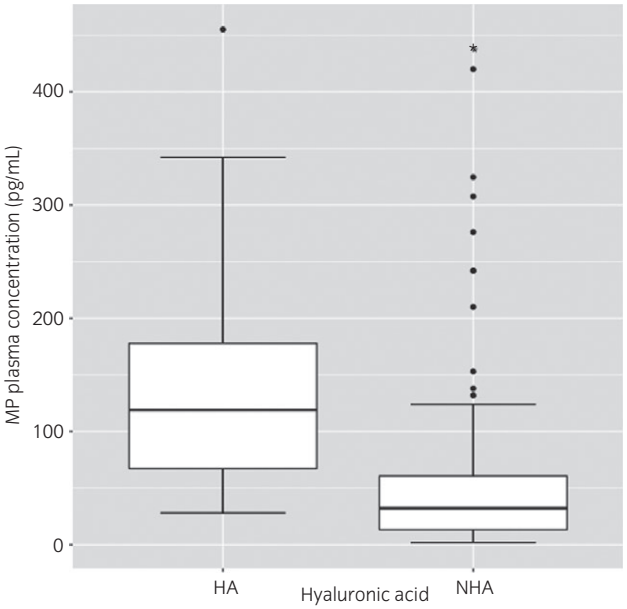

Fig 2: Box and whisker plots for plasma methylprednisolone concentrations 6 days post-injection by concomitant injection of hyaluronic acid compared with no hyaluronic acid. The addition of hyaluronic acid to the injection doubled the methylprednisolone plasma concentration.

The plasma concentration for the entire group (mean ± s.d., pg/mL) was 96 ± 104. The CJO group had a lower plasma MP concentration (53 ± 68, $P<0.05$) than the DTJ (226 ± 74) and the MFT (177 ± 154) group, and no difference was identified among the other groups evaluated. The NHA (75 ± 96) group had approximately half the MP concentration of the HA

TABLE 2: Synovial structures injected by breed

Breed	Carpal joints	Carpal sheath	Distal tarsal joints	Metacarpophalangeal joint	Medial femorotibial joint	Hyaluronic acid	No hyaluronic acid
Thoroughbred	4	0	6	4	1	7	8
Standardbred	8	4	0	5	3	14	6
Quarter Horse	24	0	1	20	6	0	41

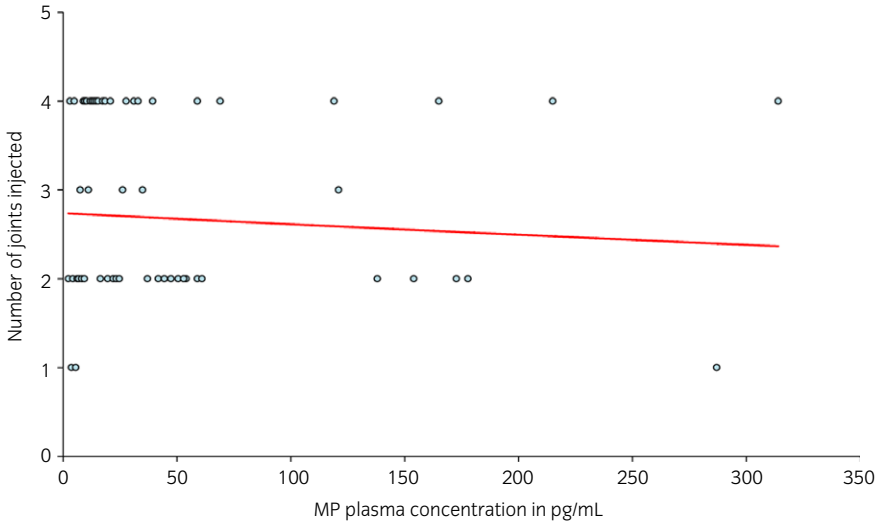

Fig 3: Number of synovial structures injected and plasma methylprednisolone concentrations 6 days post-injection (demonstrates no effect with P = 0.8).

TABLE 3: Tolerance limits or threshold values (pg/mL) for a 6-day methylprednisolone plasma concentration for two different statistical methods. The Gauss-Camp-Meidell method (P = 0.05) threshold was chosen to match the 95/95 risk of a positive test, and the Gauss-Camp-Meidell method (P = 0.01) is included to demonstrate a threshold for a risk of 1 in 100 tests

Synovial structure	N	95/95	Gauss-Camp-Meidell (P = 0.05)	Gauss-Camp-Meidell (P = 0.01)
All horses	76	487	407	791
Carpal joints	37	418	416	770
Carpal sheath	4	675	312	455
Distal tarsal joints	7	890	450	721
Metacarpophalangeal joint	34	245	199	398
Medial femorotibial joint	10	3160	636	1202
Hyaluronic acid	21	647	460	844
No hyaluronic acid	55	534	366	719

(147 ± 104, P<0.05) group. Six-day tolerance limits or thresholds generated using 95/95 or GCM (P = 0.05, P = 0.01) are presented in Table 3.

Discussion

This population study in racing horses was undertaken to evaluate the post-MP acetate administration plasma concentrations of MP under field conditions. Our findings provide guidance for practitioners when deciding upon MP acetate use in different synovial structures and demonstrate the usefulness of different statistical methods for the determination of thresholds for therapeutic substances. The results concur with and support previous findings [7] that the pharmacokinetics of MP differs depending upon which synovial structures are treated. Knych *et al.* [7] demonstrated differences in MP plasma pharmacokinetics between the antebrachial (AC) joint and the intercarpal (IC) joints, whereas our study demonstrates differences among CJO, DTJ and MFT. We were unable to repeat the findings of the previous Knych *et al.* [7] study because the AC and IC joints were commonly injected together in the practices of our investigators. Therefore, any differences between the pharmacokinetics of MP between these two specific joints would not be detected in our study design.

Metacarpophalangeal joints were associated with the most rapid clearance of MP from the plasma in this study (Fig 1), with 85% of the data falling below the LOQ (censored), and only a single horse exceeding the 21-day RMTC recommended threshold of 100 pg/mL. The high percentage of censored data in this group prevented any statistical analysis of this group. The DIP is included in the MCP group because in all cases (N = 5) in our study the two structures were injected together. Of these five horses, all were well below the 100 pg/mL threshold and 60% of the data were censored. It is likely that this joint shares the characteristics of rapid clearance from the plasma with the MCP. This may result from its considerable range of motion of 46–47 degrees of flexion/extension despite being encased in the hoof [14].

Of the joints that could be included in the statistical comparisons, the CJO had the lowest MP plasma concentrations. In the MCP, DIP and CJO joints, the low plasma concentrations of MP at 6 days post-administration may have been associated with the relative ease of the injection procedure resulting in the entire dose being deposited into the joint, the motion of the joint which could be associated with increased blood flow or high rates

of mechanical disruption of the actual MP acetate particles. In contrast, DTJ injections were associated with the highest plasma MP concentration at 6 days post-injection. This may have resulted from a larger proportion of the MP acetate being deposited outside of the joint in the subcutaneous tissues, reflux of the injectate back out through the needle track and into the subcutaneous tissue because of the anatomy of the synovial structure, or simply a lower rate of mechanical disruption of the MP acetate particles. The DTJ are characterised by minimal joint fluid volume, and even small volumes of medication injected into the joint would likely cause sufficiently increased pressure in the joint to result in significant reflux. This likelihood that a portion of MP refluxed back through the needle track is supported by the narrow standard deviation for plasma MP concentrations for DTJ, reflecting the consistency with which this higher plasma concentration is observed. In contrast, the plasma MP concentration after injection of the MFT joint was accompanied by both a high mean and standard deviation, possibly reflecting the technical difficulty of the injection procedure, or variable uptake of the MP into the infrapatellar fat pad. If any portion of the dose of MP is inadvertently delivered outside the joint/synovial cavity, a longer withdrawal can be expected. Slight movement of the horse during the MFT injection procedure could readily result in portions of the dose administered being deposited peri-articularly. The plasma MP concentrations from horses that had CS synovial structures treated had a similar profile to the distribution of MP from those with DTJ injections, suggesting a similar mechanism for medication uptake into the systemic circulation from the synovial structure. The plasma MP concentration was also higher when the MP was co-administered with HA than when it was administered alone. The large HA molecules may serve to trap the MP in the joint, or the HA may simply add volume to the injection, increasing the likelihood of reflux of the injectate into the subcutaneous space. The longer withdrawal associated with co-administration of HA has been previously observed with other corticosteroid intra-articular targeted therapies [15].

In order to provide practitioner guidance on how MP should optimally be used in practice, both the 95/95 tolerance limit and GCM method were compared (Table 3). The 95/95 tolerance limit is defined as a level with which there is 95% confidence that 95% of the population will fall below the threshold [6]. Practically, both methods determine the probability of violating a threshold given a risk level, in this case 5%. The primary differences between these statistical methodologies are the criteria which the data must meet for the method to be valid. For example, the 95/95 tolerance method requires normality and a minimum number of data points of 19 [6]. The GCM method does not require a normal distribution but does require a unimodal distribution [13].

In our MP data set, the calculated threshold varied from 257 to 3160 pg/mL, depending upon which structures were injected and which statistical method was employed to determine the threshold. The greatest discrepancy is found between the 95/95 tolerance method and the GCM ($P = 0.05$), with the MFT. Both methods theoretically carry the same risk of violating the threshold, but the 95/95 tolerance method is accompanied by a considerably higher threshold. Technically, the 95/95 method requires a sample size of at least 19 animals [7], and the sample size for the MFT in this study included only 10 horses. This exemplifies why it is important that the most appropriate statistical methodology for the data be employed in threshold determination. In the case of a sample size less than 19, clearly, the 95/95 tolerance level is inappropriate.

This study provides several important guidelines for veterinarians using targeted joint therapy with MP acetate in equine athletes that perform in a regulated environment. First, the use of MP in MCP, DIP and CJO joints without the concurrent administration of HA is likely to be associated with a shorter withdrawal time before a competitive event than other applications of the product. Second, caution should be used in the administration of MP to MFT joints because the plasma concentrations of MP are highly variable when MP acetate is administered into this synovial structure. Efforts to improve the accuracy of MFT injection should be made, including sedation of the patient and injection by ultrasound guidance to ensure that the entire dose is deposited within the joint pouch. Finally, DTJ and CS require a longer withdrawal for MP acetate administration than when used in other joints, likely as a result of their anatomy, and the potential for medication reflux out of the synovial structure after injection. For existing thresholds, specific withdrawal times before competition for MP acetate injection into disparate synovial structures cannot be recommended based on our findings. Additional studies are warranted to provide this information.

Authors' declaration of interests

Professor George Maylin provides drug testing services for racing jurisdictions. Professor Thomas Tobin and Dr Clara Fenger have testified frequently as experts in matters involving medication regulation.

Ethical animal research

The authors have provided confirmation that research ethics committee oversight was not required: the study was performed on material collected during clinical procedures and results were shared with owners or trainers. Owners or trainers gave consent for their animals' inclusion in this study.

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Authorship

All authors contributed to study design, study execution and gave their final approval of the manuscript. J. Machin, W. Duer, C. Fenger, G. Maylin and T. Tobin also contributed to data analysis and interpretation, and preparation of the manuscript.

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