TITLE
Bayesian-based withdrawal estimates using pharmacokinetic parameters for two capsaicinoid-containing products administered to horses.
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RUNNING TITLE
Withdrawal Estimates for Capsaicinoids in Horses

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ABSTRACT

Capsaicinoids deter horses from chewing on bandages and are applied topically to provide analgesia to musculoskeletal injuries. They are banned during competition due to their nerve blocking properties. The pharmacokinetics of oral (PO) and direct gastric administration via nasogastric tube (NG), or topical (TOP) administration of two capsaicinoid-containing products were investigated, and the withdrawal times required prior to competition were estimated. Capsaicin (CAP) and dihydrocapsaicin (DCAP) were quantified in plasma, and both compounds were best described by a delayed absorption two compartment elimination model following PO administration, and by a first order absorption one compartment elimination model following TOP administration. Capsaicin and DCAP could not be quantified in most samples following NG administration. Following PO administration, the time to maximum plasma concentration (Tmax) for CAP and DCAP was 0.25 (0.08 – 0.50) h. Following TOP application, the Tmax for CAP and DCAP was 4 (2 to 6) and 5 (3 to 12) h, respectively. By 8 hours post-PO administration, and 36 hours post-TOP application, CAP and DCAP were below the lower limit of quantification. Capsaicin and DCAP were not detected in urine samples. Withdrawal times were predicted using the 99.99% credibility interval limits of the pharmacokinetic parameters calculated with Bayesian estimation.

KEY WORDS
Bayes theorem, capsaicin, dihydrocapsaicin, horses, oral administration, topical administration

ABBREVIATIONS
AUC\textsubscript{0}\textsuperscript{\infty} area under the curve from time zero to infinity
CAP capsaicin
Cl/F apparent clearance without adjusting for bioavailability
Cmax maximum measured concentration
INTRODUCTION

Capsaicinoids are compounds isolated from the fruits of hot pepper plants. The crude dark extract, oleoresin, contains over 100 distinct volatile compounds, and is marketed in many products with widely varying degrees of efficacy. The basic chemical structure consists of a vanilloid ring and various alkyl side chains that define the specific analogs. Capsaicin (8-methyl-n-vanillyl-6-nonenamide) is a pure white crystalline material (Cordell & Araujo, 1993), and is the most abundant active compound isolated from this mixture (C. A. Reilly, Crouc, Yost, & Fatah, 2001; Christopher A. Reilly & Yost, 2006). Other prevalent compounds are dihydrocapsaicin (N-[(4-hydroxy-3-methoxyphenyl) methyl]-8-methyl-nonanamide), nonivamide, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin (C. A. Reilly et al., 2001; Christopher A. Reilly & Yost, 2006).

Of the many culinary spices, the capsaicinoids appear to have the most ancient record of use (Govindarajan, 1985). These substances produce the characteristic “hot” sensations associated with the ingestion of spicy foods by binding to the transient receptor potential (TRP) family of ion channels. Capsaicin (CAP) and dihydrocapsaicin (DCAP) are highly selective for
the TRP vanilloid subtype 1 (TRPV1) receptor, which is preferentially expressed on the small-diameter sensory neurons responsible for transmitting painful sensations (Caterina et al., 1997; Pedersen, Owssianik, & Nilius, 2005; Szallasi & Appendino, 2004). Capsaicinoids are unique among naturally occurring irritant compounds because initial exposure of nociceptor terminals to these compounds produces excitation of the neuron, the perception of pain, and the local release of inflammatory mediators. The excitation soon subsides, however, and is followed by a prolonged refractory period, termed desensitization, during which the previously excited neurons are no longer responsive (Bode & Dong, 2011; Holzer, 1991). In contrast to local anesthetics, capsaicinoids do not block motor function (Conway, 2008), which is an advantage in the management of pain in limbs and has been exploited therapeutically.

Topical capsaicinoid-containing products have been in clinical use by humans for many years and are available over the counter. Several studies have shown that low concentrations (0.025% to 0.1%) of CAP applied topically require repeated administration for maximum efficacy (Chrubasik, Weiser, & Beime, 2010; Kosuwon, Sirichatiwapee, Wisanuyotin, Jeeravipolvav, & Laupattarakasem, 2010). A single application of a patch containing high concentrations of CAP (8%) was able to produce similar results (Malmberg et al., 2004). Intractable and neuropathic pain have been treated topically with 5 to 10% CAP (Jones, Moore, & Peterson, 2011; Kennedy et al., 2010; McCormack, 2010; Robbins et al., 1998; K. Roberts, Shenoy, & Anand, 2011; Simpson et al., 2010).

Horses are exposed to capsaicinoids by the oral and topical routes. Capsaicinoid-containing products (e.g. cayenne pepper) are commonly applied to the surface of bandages and the surrounding environment to deter horses from destructive chewing. Capsaicinoid-containing creams and lotions are also available over the counter for topical use on the horse. Topical application of a commercially available capsaicinoid-containing product (EquiBlock, 0.025%) over the palmer digital nerve provided measurable improvement in a reversible lameness model for at least 4 hours after treatment (Seino, Foreman, Greene, Goetz, & Benson, 2003), and the presence of capsaicinoids in samples collected for drug testing of equine athletes is prohibited by many regulatory agencies. The time at which exposure must be discontinued prior to competition to avoid a drug violation, i.e. the withdrawal time, for these products is unknown.

To estimate withdrawal times, the pharmacokinetics must be available. No studies have investigated the pharmacokinetics (PK) of capsaicinoids in the horse following their ingestion,
and very limited PK data is available for products applied to the skin (You et al., 2013; Zak et al., 2018). The primary purpose of this study was to investigate the PK of two capsaicinoids, CAP and DCAP, found in commercially available products frequently used around or on horses and to suggest appropriate withdrawal times for these products prior to competition using a novel Bayesian approach.

MATERIALS AND METHODS

Animals.

Studies were conducted following protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC 803452) and are in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals and Guide for the Care and Use of Laboratory Animals. All horses underwent routine dental and foot care and scheduled administration of appropriate vaccinations and de-worming agents. The horses were no longer actively racing. Based on physical examination and routine blood work, all horses were in good health. Horses were housed on pasture and brought into a temperature-controlled facility 2 days before the study period. Studies were performed in the months of November to March and temperatures were maintained between 60 and 65°F. Horses were fed approximately 1 kg of grain in the morning and late in the afternoon; grass hay and water were available ad libitum. Horses were pasture fit. The 6 mares and 6 geldings used for the study were 8 Thoroughbreds, 3 Standardbreds, and 1 Warmblood, weighing 560 ± 57 kg, and ranging from 4 to 13-years old (mean 8 years).

Oral (PO) administration.

A commercially available cayenne pepper powder (Ground cayenne, McCormick & Co, Hunt Valley, MD) was administered to 6 horses PO (3 Thoroughbred Mares, 1 Standardbred Mare, 2 Standardbred Geldings). The powder (7.5 g) containing 48.3 ± 4.5 µg/kg of CAP and 27.0 ± 2.5 µg/kg of DCAP was suspended in 30 mL of molasses, administered via an oral dosing syringe. Horses reacted mildly with lip smacking for a period lasting no more than 5 minutes.

Nasogastric (NG) administration.

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Another 6 horses received cayenne pepper powder via a NG tube (2 Thoroughbred Mares, 3 Standardbred Geldings, 1 Warmblood Gelding). For NG administrations, the same amount (7.5 g) was suspended in 500 mL of water and administered via a NG tube directly into the stomach. The powder contained 46.2 ± 4.4 μg/kg CAP and 25.9 ± 2.5 μg/kg DCAP. The tube and funnel were flushed with an additional 500 mL of water.

Topical (TOP) application.

A capsaicinoid-containing ointment (0.025% CAP, Equi-Block™, Miracle Corp, Moraine, OH) was administered using a randomized cross-over design to the same 6 horses that received the cayenne pepper powder by the PO route. The anterior and posterior region of the left carpal joint was clipped of hair 10 cm above and below the center of the joint. Fifteen grams of ointment were placed on a Telfa pad (3 x 4 inches, Covidien, Minneapolis MN), and the ointment was evenly distributed between 2 Telfa pads. The pads were placed on the anterior and posterior surfaces of the carpus so that the ointment was maintained between the pad and skin. They were held in place for 24 hours with a bandage composed of a layer of cast padding and Elasticon (Johnson and Johnson, Skillman NJ). The surface area covered by the Telfa pads was 155 cm², yielding an average (SD) CAP dose of 31 ± 9 μg/cm² and DCAP dose of 21 ± 7 μg/cm².

Blood and urine sampling.

A 14-F catheter (Angiocath, Becton Dickinson, Sandy, UT) was placed aseptically into the jugular vein for collection of blood samples. Following drug administration, blood samples were collected into sodium fluoride/potassium oxalate-containing blood tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ). Collection times are shown in Table 1. Blood samples were stored on ice until the plasma was harvested by centrifugation (2500–3000 rpm or 776–1318 g) at 4 °C for 15 min. Harvesting of plasma took place within 15 to 20 minutes of sample collection and 3 mL aliquots of plasma were immediately frozen and stored at -80 °C until analysis.

A sterile indwelling 24-F self-retaining catheter (Foley Catheters, CR Bard Inc., Covington, GA) was placed in the bladder of 5 female horses (TOP n = 4, PO n = 1) and attached to a drainage bag (Bard Center Entry Urinary Drainage Bag, CR Bard Inc., Covington,
GA) for continuous collection of urine. Prior to placement, the vulva was washed with surgical soap and rinsed with sterile water. The total volume of urine excreted was collected and measured at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, and 24 h after drug administration. A sample was also collected by catheterization at 36, 48 and 72 h post-administration from some animals. One gelding (TOP) was fitted with a urine collection bag for the collection of urine at the same time points listed above. Aliquots of urine (3 mL each) were immediately frozen at -80°C, and only thawed once prior to analysis.

Quantification of CAP and DCAP.

Capsaicin and DCAP were quantified in equine plasma and urine using ultra high-performance liquid chromatography-tandem mass spectrometry within 3 months of collection to ensure there was minimal degradation of the analytes during storage (You et al., 2013). In brief, analytes were recovered by liquid-liquid extraction using methyl tert-butyl ether, separated on a 1.9 µm C₁₈ reverse-phase column and analyzed by positive electrospray ionization mode on a triple quadrupole mass spectrometer (Thermo TSQ Ultra) with selected reaction monitoring (SRM). The transitions for CAP and DCAP quantification were m/z 306→137 and m/z 308→137, respectively. Deuterium-labeled CAP was used as an internal standard for both analytes. The lower imits of quantification (LLOQ) for CAP and DCAP for this study were 2.5 and 5.0 pg/mL, respectively.

The concentration of CAP and DCAP in two bottles of cayenne pepper powder measured using liquid chromatography coupled to mass spectrometry (You et al., 2013) were not consistent. One bottle contained 3,411 ± 392 µg/g CAP and 1,917 ± 403 µg/g DCAP. The other bottle contained 3,654 ± 247 µg/g CAP and 2,007 ± 136 µg/g DCAP. The amount of CAP and DCAP in two batches of the capsaicinoid-containing ointment was estimated using liquid chromatography coupled to mass spectrometry (You et al., 2013) to be 250 ± 20 and 168 ± 22 µg/g, and to be 383 ± 96 and 266 ± 75 µg/g, respectively.

Pharmacokinetic analysis.

Compartmental analysis was used to describe the disposition and elimination of the plasma concentration versus time data for CAP and DCAP obtained following PO and TOP administrations. Plasma concentration versus-time curves from each horse were modeled using
nonlinear regression with a mathematical modeling software package (WinSAAM, University of Pennsylvania, Kennett Square PA). Visual inspection of the PO and TOP data suggested that the data would fit a model with first order absorption and either 1-or 2-compartment elimination. The best fit was obtained by minimization of the fractional standard deviation of the micro rate constants and the convergence of the predicted plasma concentration curves to the observed data (Figure 1).

Bioavailability (F) was not determined for CAP and DCAP in the horse because it is not ethical to administer these substances intravenously to conscious animals and could be dangerous in horses (Jancso, Jancso-Gabor, & Szolcsanyi, 1967; Yaksh, Farb, Leeman, & Jessell, 1979). Thus, the apparent volume of distribution (Vc/F) was an estimated parameter of the model. Apparent clearance (Cl/F) was calculated as:

\[ \text{Cl/F} = \frac{D}{\text{AUC}_0^{\infty}} \]

where the dose is the amount of CAP or DCAP administered. The total area under the plasma concentration versus time curve from 0 to infinite time (\(\text{AUC}_0^{\infty}\)) was calculated by non-compartmental analysis (Phoenix 64 version 6.4.0.768, Cary NC, USA) using the linear-log trapezoid method (Gabrielsson & Weiner, 2006). Maximum plasma concentration (\(C_{\text{max}}\)), and the time to reach the maximum plasma concentration (\(T_{\text{max}}\)) were determined directly from the data. Pharmacokinetic parameters are expressed as median (min, max) and plasma concentration data are expressed as mean (SD) unless otherwise specified.

Withdrawal time predictions using 99.99% Credibility Intervals

For estimating the mean and the 99.99% credibility interval (99.99% Cred. I.) of the PK parameters, Bayesian regression using the Metropolis-Hastings algorithm with non-informative priors (flat prior) in STATA 14 (MP, StataCorp, College Station TX) was implemented (G. O. Roberts & Rosenthal, 2001). There were 10,000 burnin simulation steps followed by 10,000 mcmc simulation steps. For generating the average profile for the PO and TOP administration, the mean of all adjustable parameters from each model was used. For estimating the fastest elimination profile, the lower 99.99% Cred. I. limit for the delay time and the upper 99.99% Cred. I. limit for Vc/F, and all micro-rate constants of the model were used. The inverse was done for estimating the slowest elimination profile.

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Statistics.

Significant differences in PK parameters between CAP and DCAP were determined by a paired t-test when the data were normally distributed (STATA 14, MP, StataCorp, College Station TX), and significance was established when p < 0.05. Normality of the parameters was assessed using the Shapiro-Wilk normality test. For non-normal parameters (TOP DCAP $K_\alpha$, PO DCAP Cmax) the data were log transformed to yield normally distributed data prior to performing the paired t-test. Parameters that could not be normalized by log transformation (Tmax) were compared using the Wilcoxon signed-rank test.

RESULTS

Pharmacokinetics of CAP and DCAP following PO administration.

Following the PO administration of cayenne pepper, plasma concentration versus time curves of CAP and DCAP were best described by a 2-compartment elimination model with a fixed $K_a$ and $Vc/F$ following a DT (Figure 2 dotted lines) with the PK parameters shown in Table 2. Capsaicin and DCAP were rapidly absorbed and were measured in all 6 horses by 2 minutes (Figure 2, Table 1). Capsaicin and DCAP were also rapidly eliminated from plasma. Capsaicin was no longer detected in 1 horse by 4 h, in another 3 horses by 6 h, and could not be detected in any horse at 8 h. Dihydrocapsaicin was no longer detected in any horse by 6 h (Figure 2, Table 1). Neither capsaicinoid was detectable in any of the urine samples collected following the PO administration of cayenne pepper.

Using Bayesian regression and the 99.99% credibility interval of the PK parameters, the withdrawal time required for CAP and DCAP to fall below the LLOQ was predicted for the average, slowest eliminating, and fastest eliminating theoretical horse (Figure 2 solid lines; Table 2).

Pharmacokinetics of CAP and DCAP following NG administration.

Following the NG administration of cayenne pepper, CAP and DCAP were measured in only a small number of the plasma samples obtained from each horse, and PK modeling could not be performed (Figure 3).
Pharmacokinetics of CAP and DCAP following TOP application.

Following TOP application of a capsaicinoid-containing ointment, plasma concentration versus time curves of CAP and DCAP were best described by a first order absorption one compartment elimination model (Figure 4 dotted lines) with the PK parameters shown in Table 2. Both capsaicinoids were rapidly absorbed through the skin. Capsaicin and DCAP were measured in plasma by 15 minutes in 5 and 2 horses, respectively (Figure 4, Table 1). By 45 min post-application, CAP was measured in all 6 horses, and by 2 hours post-application, DCAP was measured in 5 horses. Dihydrocapsaicin was detectable, but remained below the LLOQ in all samples collected from 1 horse.

Elimination was slower following TOP application than following PO administration. Capsaicin was no longer detected in 1 horse at 24 h, in 2 more horses at 48 h, and by 72 h could not be detected in any horse. DCAP was no longer detected in 2 horses at 24 h, 2 more horses at 48 h, and by 72 h could not be detected in any horse (Table 1). CAP and DCAP were not detectable in any of the urine samples collected following the TOP application of the capsaicinoid-containing ointment.

Using Bayesian regression and the 99.99% credibility interval of the PK parameters, the withdrawal time required for CAP and DCAP to fall below the LLOQ was predicted for the average, slowest eliminating, and fastest eliminating theoretical horse (Figure 4 solid lines, Table 2).

DISCUSSION

This is the first study to model the PK of two capsaicinoid-containing products in the horse, cayenne pepper and Equi-Block™. Capsaicinoids are prohibited in equine athletes during competition due to their documented analgesic properties when used topically on horses (Seino et al., 2003). Analytical tests cannot distinguish between topical and oral administrations, thus horse caretakers must discontinue exposure to both types of products within an appropriate time frame prior to competition. The data generated in this study describes the PK parameters for CAP and DCAP and provides models that can be used to estimate when to eliminate these products prior to competition.

No published studies characterizing the elimination of CAP and DCAP from the horse following PO or NG administration were identified for comparison with the present study. Several studies have investigated the PK of these compounds following oral gavage to rats.
When a CAP and DCAP mixture (85% CAP, 15% DCAP) was administered by oral gavage at 14 mg/kg, 85% of the administered dose was eliminated from the gastrointestinal tract within 3 hours, and less than 10% of the total administered dose could be recovered in the feces 48 hours after the administration (Kawada, Suzuki, Takahashi, & Iwai, 1984). Metabolites could not be identified in the gastrointestinal contents sampled directly suggesting that absorption was the primary mechanism for the removal of CAP and DCAP from the gastrointestinal tract of rats.

In another study in rats, 30 mg/kg of CAP was administered by oral gavage, and a Cmax of 1.09 ± 0.18 µg/mL was measured in serum at the first time point collected, which was 1 hour after its administration (Suresh & Srinivasan, 2010). Both the dose and Cmax measured in this study were ~600-fold greater than the dose and Cmax measured by the present study in horses (CAP dose = 48.3 ± 4.5 µg/kg, Cmax = 1543 (884 – 2188) pg/mL, Tmax = 0.25 (0.08 – 0.25) h).

Interestingly, the NG administration of cayenne pepper to horses, which could mimic oral gavage administration to rats, resulted in plasma concentrations of CAP and DCAP that were at or below the LLOQ.

Three possible explanations for the different plasma concentrations observed in horses following the PO and NG routes are proposed. 1) Absorption of capsaicinoids by the horse may primarily occur aboral to the stomach via the mucous membranes, with poor absorption after nasogastric administration by the stomach and distal gastrointestinal tract. Mucosal absorption is suspected to occur due to the very rapid observation of CAP and DCAP in plasma following oral administration (within 1 min). 2) The presence of a significant first pass effect may be present in the horse, as has been reported in rats (Donnerer, Amann, Schuligoi, & Lembeck, 1990; Kawada et al., 1984), which greatly reduced the detection of CAP and DCAP after NG administration.

The current study did not evaluate fecal concentrations of CAP and DCAP or measure metabolites of these substances in any matrix. 3) It must be considered that the amount of CAP and DCAP delivered by the NG route may have been less than expected due to adsorption to the funnel and nasogastric tube. While it is assumed a small amount of adsorption would not have had a significant effect on the total amount delivered due to visual inspection of the apparatus and the rapid delivery of the suspension within a large volume (500 mL followed by flushing with 500 mL), it is a limitation of the study that the concentrations of CAP and DCAP were not measured following the passage of the suspension through the delivery apparatus.
The administration of an intravenous dose of CAP and DCAP was not performed due to ethical and safety concerns of doing so in a conscious horse. No previous studies were identified in any species in which capsaicin was administered intravenously without anesthesia. When capsaicin was administered intrathecally to conscious rats, a violent reaction was seen lasting for 10 to 15 minutes (Yaksh et al., 1979). In another publication, capsaicin was administered both subcutaneously and intraperitoneally under light plains of ether anesthesia. Despite general anesthesia, a reflex apnea occurred in some rats and artificial respiration was necessary (Jancso et al., 1967). Therefore, bioavailability and a definitive explanation for the difference in the plasma concentrations observed between PO and NG administration to horses cannot be determined by this study and may warrant future investigation by other means, such as measuring CAP and DCAP in feces or increasing the dose administered via the NG route.

For substances regulated for doping control purposes, veterinarians need to consider all possible routes of exposure to appropriately advise their clients how to avoid a violation. Capsaicinoids are present in many topical over-the-counter products marketed for horses and humans. The absorption of a substance by the skin into systemic circulation depends on many factors, one of which is the lipophilicity of the compound. The octanol/water partition coefficient (LogP) for CAP was calculated as 3.75 (Csizmadia, Tsantili-Kakoulidou, Panderi, & Darvas, 1997; Klopman, Li, Wang, & Dimayuga, 1994; Viswanadhan, Ghose, Revankar, & Robins, 1989), which falls in the ideal LogP range for skin penetration which is 1 to 4 (Riviere, 2001).

The topical application of two capsaicinoid-containing products to horses has been studied previously. Zak et al. applied 15 g of Horse Gel (0.1% CAP, Eclipse Biofarmab, Sweden) to each thoracic limb by massaging it into the skin for 5 min while wearing Latex gloves (Zak et al., 2018). The limbs were then wrapped with a polar fleece bandage for 12 h, and the procedure was repeated for 5 days. Blood samples were collected before and after the last application with the first post-administration sample collected 12 h after the application. CAP and DCAP were not detected above the LLOQ (0.5 pg/mL and 1.0 pg/mL, respectively) in any of the plasma or serum samples collected.

In contrast, CAP and DCAP were measured for up to 24 h in a pilot study performed in a single horse (You et al., 2013), and was measured in the present study for up to 48 h in 3 of the 6 horses. Several study characteristics may explain the differing plasma concentrations observed.
in these studies. 1) The product in the study by Zak et al. “was applied to each thoracic limb from the level of the carpal joint to the pastern joint”. This is a much larger and undefined surface area than used in the present study, potentially resulting in a lower dose per square centimeter despite the higher amount of CAP reported to be in the product they applied (0.1% instead of 0.025%). 2) The skin was clipped of hair in the present study, but not in the study by Zak et al. Hair will impede the interaction of the product with the skin and could explain why they did not detect CAP. 3) The leg was wrapped for 24 h in the present study instead of 12 h, which increased the length of exposure to the product. 4) The bandages used in the studies were different. While both are water permeable, the degree of water permeability may be different and could have had an effect on absorption. 5) Different products were applied, and the excipients in these products may greatly alter the absorption of CAP and DCAP. 6) Zak et al. did not measure the concentration of CAP and DCAP in the product applied and relied on the manufacturer’s reported CAP concentration (0.1%). It is possible there are batch differences as was observed in the present study. Other possible reasons may exist, and it is not surprising given the number of variables affecting the absorption of topical products that there were differences between the studies. Importantly, the differences between these studies highlight that withdrawal estimates provided by any study can only be applied reliably if the conditions of the study are closely reproduced.

CAP and DCAP were not detected in any of the urine samples collected following either route of administration. This is consistent with previous studies in rats demonstrating that a very small percentage of CAP (0.095%) and DCAP (8.7%) were measured unchanged in the urine following oral gavage of a much larger CAP dose (30 mg/kg versus 48.3 ± 4.5 μg/kg) or DCAP dose (20 mg/kg versus 27.0 ± 2.5 μg/kg) (Kawada & Iwai, 1985; Suresh & Srinivasan, 2010). Thus, it is suggested that regulatory bodies test plasma or serum to identify the inappropriate use of capsaicinoids.

Capsaicinoids are not permitted in samples collected from horses competing in pari-mutuel racing and other regulated sporting events. Regulated substances need to be discontinued prior to competition and the withdrawal time is the time it takes for CAP and DCAP to fall below the ability of the drug testing laboratory to confirm these substances according to industry standards ie. the limit of confirmation (LOC). The guidelines established by the Association of Official Racing Chemists and ILAC-G7 (Accreditation Requirements and Operating Criteria for
Horseracing Laboratories) are the gold standard and are followed by most equine drug testing laboratories. When following these guidelines, the LOC is usually equivalent to the LLOQ for a quantitative method validated to accurately measure the substance, however some therapeutic medications are permitted below a screening limit or threshold established to ensure the safety of the horse and the integrity of the sport. Several approaches have been used to recommend “safe” withdrawal times using data from PK studies.

In one approach, the time at which the reported concentration falls below the regulatory limit for all animals in the study is called the detection time (Toutain, 2010). To extrapolate the detection time to the population, a safety factor multiplied by the detection time was recommended so that individuals that eliminate the drug more slowly than the animals in the PK study will be less likely to have a violation. Using Monte Carlo simulations, a safety factor of 2.4 is predicted to be acceptable for the majority of drugs, dosing regimens and horses (Toutain, 2010).

Another approach is to define a threshold for drugs used therapeutically in either plasma or urine above which the substance is prohibited during competition due to known effects of the drug at higher concentrations or due to the desire to prohibit the use of a drug within a certain time frame prior to competition. When the later approach is used, a 95/95 upper tolerance limit can be calculated from data collected at the time point at which the industry decides the drug should be discontinued, and that value is used as the threshold for the drug (RMTC, 2016). The recommended withdrawal time becomes the time point used to calculate the threshold value. This approach accounts for the variability inherent in the study population and adds a safety factor by calculating the 95/95 upper tolerance limit, however if a substance must be below the LLOQ of the analytical method, as is the case for CAP and DCAP, this method cannot be applied.

In this study, a new approach was employed to provide withdrawal time estimates for CAP and DCAP. To the authors’ knowledge, this is the first study to use a Bayesian approach to generate 99.99% credibility intervals for the estimated PK parameters, and to apply the average, upper and lower limits of those intervals to generate the predicted average, slowest and fastest elimination profiles for the drug of interest i.e. CAP and DCAP following PO and TOP administration. There are several advantages to this approach. First, it can be used for any substance regardless of whether a threshold exists. Second, by using this approach, direct
visualization of the estimated plasma concentrations for the average, slowest and fastest eliminating horses expected to exist in the population provides veterinarians with an intuitive way to assess the risk of an overage if a shorter or longer withdrawal period is employed by their client for a particular horse. The astute client will be able to see the amount of risk inherent in using the substance and in consultation with their veterinarian make a decision whether to enter the horse to compete following a defined medication regimen. Third, instead of providing a single withdrawal recommendation, a range is provided (average, slowest, fastest) that reflects the true range of possible withdrawal times that could be successfully employed. By presenting the data in this manner, veterinarians and their clients will be able to understand that the withdrawal time required is not a specific value but is an estimate and is horse and situation specific.

A major limitation is that the withdrawal times predicted using any of the methods presented above are invariably derived from a small number of animals. The performance of population studies to validate the applicability of these withdrawal time estimates are needed to ensure their reliability. Since these studies are expensive and do not exist, many racing jurisdictions provide for a grace period following a change in the way a therapeutic substance is tested for and regulated. This allows for identification of any unanticipated differences between the withdrawal estimate derived from the study population and the withdrawal time needed in the racehorse population.

**CONCLUSIONS**

Capsaicin and DCAP are rapidly absorbed and eliminated by the PO and TOP routes, and removal of horses from both types of exposure prior to competition is necessary. The PK models developed provide a novel way for veterinarians and their clients to visually determine an appropriate withdrawal interval for horses exposed to these capsaicinoid-containing products provided the horse was exposed to these products at the same or lower doses, and the LLOQ is the same as that reported herein. In the current study, CAP and DCAP were below the LLOQ by 8 hours following PO administration, and by 36 hours following TOP application, however the amount of variability in the data following the TOP application suggests that a longer withdrawal time may be prudent as reflected in the profiles generated using the 99.99% credibility intervals.
ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTION STATEMENT

MAR and LRS designed the study. MAR administered the products and coordinated the sample collection. YY performed the sample analysis. DS, RB, MAR, and LRS analyzed the data. All authors contributed to the manuscript and have read and approved the final manuscript.

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Table 1. Mean (SD) capsaicin (CAP) and dihydrocapsaicin (DCAP) plasma concentrations (pg/mL) by route. An asterisk indicates that the concentration in some horses was above the LOD but below the LLOQ and was not included in the calculation of the Mean (SD).

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<td>ND</td>
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<td>0.08</td>
<td>1118 ± 416</td>
<td>141 ± 50</td>
<td>BLQ</td>
<td>ND</td>
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<tr>
<td>0.25</td>
<td>1564 ± 451</td>
<td>405 ± 209</td>
<td>3 ± 4</td>
<td>BLQ</td>
<td>3 ± 1*</td>
<td>BLQ</td>
</tr>
<tr>
<td>0.50</td>
<td>599 ± 304</td>
<td>199 ± 112</td>
<td>3 ± 2*</td>
<td>BLQ</td>
<td>8 ± 4</td>
<td>BLQ</td>
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<td>0.75</td>
<td>272 ± 152</td>
<td>101 ± 41</td>
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<td>BLQ</td>
<td>13 ± 7</td>
<td>BLQ</td>
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<tr>
<td>1.00</td>
<td>207 ± 137</td>
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<td>16 ± 8</td>
<td>5 ± 2*</td>
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<td>2.00</td>
<td>42 ± 27</td>
<td>17 ± 9*</td>
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<td>ND</td>
<td>23 ± 9</td>
<td>8 ± 4*</td>
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<tr>
<td>3.00</td>
<td>12 ± 4</td>
<td>6 ± 3*</td>
<td>ND</td>
<td>ND</td>
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<td>8 ± 2*</td>
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<tr>
<td>4.00</td>
<td>3 ± 2*</td>
<td>BLQ</td>
<td>ND</td>
<td>ND</td>
<td>23 ± 10</td>
<td>8 ± 3*</td>
</tr>
<tr>
<td>6.00</td>
<td>2.5*</td>
<td>ND</td>
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<td>20 ± 10</td>
<td>8 ± 4*</td>
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<tr>
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<td>ND</td>
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<td>15 ± 8</td>
<td>6 ± 3*</td>
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<tr>
<td>10.00</td>
<td>ND</td>
<td>ND</td>
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<td>11 ± 7</td>
<td>5 ± 3*</td>
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<tr>
<td>12.00</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>11 ± 7*</td>
<td>5 ± 3*</td>
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<tr>
<td>16.00</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>7 ± 5*</td>
<td>BLQ</td>
<td></td>
</tr>
<tr>
<td>20.00</td>
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<td>ND</td>
<td></td>
<td>4 ± 4*</td>
<td>BLQ</td>
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<td>ND</td>
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</table>

*PO = oral, NG = nasogastric, TOP = topical, BLQ = detected below the lower limit of quantification in some horses, ND = not detected, Blank = no sample collected.

Table 2. Pharmacokinetic parameter estimates for capsaicin (CAP) and dihydrocapsaicin (DCAP) following a single administration of cayenne pepper per os (PO) to the horse (n = 6, 7.5 g cayenne pepper per horse = 48.3 ± 4.5 µg/kg CAP and 27.0 ± 2.5 mg/kg DCAP), or following
a single topical application (TOP) of a capsaicinoid-containing ointment to the horse (EquiBlock, CAP dose of $31 \pm 9 \, \mu g/cm^2$ and DCAP dose of $21 \pm 7 \, \mu g/cm^2$; $n = 6$). Median and range are presented, except for DT, Vc/F, and the micro-rate constants for which the Bayesian mean and 99.99% credibility interval are presented.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PO</th>
<th>TOP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAP</td>
<td>DCAP</td>
</tr>
<tr>
<td>$C_{max}$ (pg/mL)</td>
<td>1543 (884 – 2188)</td>
<td>366 (236 – 817)</td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>0.25 (0.08 – 0.25)</td>
<td>0.25 (0.25 – 0.5)</td>
</tr>
<tr>
<td>$AUC_0^\infty$ (h•pg/mL)</td>
<td>857 (453 – 1210)</td>
<td>276 (135 – 341)</td>
</tr>
<tr>
<td>Cl/F (L/h)</td>
<td>3.0 (2.2 – 5.7) $^{10^4}$</td>
<td>5.4 (4.4 – 11.1) $^{10^4}$</td>
</tr>
<tr>
<td>DT (min)</td>
<td>6.1 (4.2 – 7.7) *</td>
<td>8.3 (7.3 – 9.3) *</td>
</tr>
<tr>
<td>Vc/F (L)</td>
<td>7.0 (0.2 – 12.0) $^{10^6}$</td>
<td>7.0 (0.2 – 12.0) $^{10^6}$</td>
</tr>
<tr>
<td>$K_\alpha$ (1/h)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>$k_{21}$ (1/h)</td>
<td>2.1 (0.7 – 3.7)</td>
<td>3.6 (2.3 – 5.0)</td>
</tr>
<tr>
<td>$k_{12}$ (1/h)</td>
<td>2.8 (0.1 – 5.5) + *</td>
<td>12.9 (2.2 – 22.2) *</td>
</tr>
<tr>
<td>$k_{10}$ (1/h)</td>
<td>5.2 (2.4 – 8.4) *</td>
<td>8.6 (5.1 – 12.6) *</td>
</tr>
<tr>
<td>$t_{1/2h\alpha}$ (h)</td>
<td>0.13 (0.08 – 0.29) *</td>
<td>0.08 (0.06 – 0.14) *</td>
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<tr>
<td>$\downarrow$ LLOQ (h)</td>
<td>4.3 (2.5, 7.4) *</td>
<td>3.0 (2.0, 3.7) *</td>
</tr>
</tbody>
</table>

$C_{max}$ = maximum measured CAP or DCAP plasma concentration; $T_{max}$ = time at maximum measured plasma concentration; $AUC_0^\infty$ = area under the plasma concentration versus time curve; Cl/F = apparent clearance; $DT$ (min) = time delay prior to transfer of CAP or DCAP from the time delay compartment to compartment 1; Vc/F = apparent volume of distribution; $K_\alpha$, $k_{12}$, $k_{21}$, and $k_{10}$ = micro-rate constants; $\downarrow$ LLOQ = time at which the average (fastest – slowest) calculated 99.99% credibility interval profiles fall below the lower limit of quantification for CAP (2.5 pg/mL) and DCAP (5.0 pg/mL); NA = not applicable. + 98% Cred.Int. * significant difference between CAP and DCAP ($p < 0.05$)
Figure 1. Best-fit models for capsaicinoid concentration versus time data. (A) Following PO administration, the data were best fit with a delay time compartment followed by a fixed rate of absorption and two compartment elimination. (B) Following TOP application, the data were best fit by a first order absorption one compartment elimination model. Dose was the amount of CAP or DCAP (pg) administered, $K_\alpha$ was the absorption rate constant, $k_{12}$ and $k_{21}$ were fractional rate constants between compartment 1 and compartment 2, and $k_{10}$ was the elimination rate constant from compartment 1.

A.

\[ \begin{align*}
  &K_\alpha \\
  &\text{Delay Time Compartment} \\
  &k_{12} \quad k_{21} \\
  &k_{10} \\
\end{align*} \]

B.

\[ \begin{align*}
  &K_\alpha \\
  &\text{Dose} \\
  &0 \quad 1 \\
  &k_{10} \\
\end{align*} \]

Figure 2. Capsaicin (CAP: A) and dihydrocapsaicin (DCAP: B) plasma concentration versus time data observed (markers) and predicted (dotted lines) following the oral (PO) administration of cayenne pepper ($n = 6$, 7.5 g cayenne pepper per horse = $48.3 \pm 4.5 \mu g/kg$ CAP and $27.0 \pm 2.5 \mu g/kg$ DCAP). The 99.99% credibility interval is contained between the outer solid lines, and
the central solid line represents the average predicted profile. The horizontal line represents the lower limit of quantification (LLOQ) for the analytical method employed (CAP = 2.5 pg/mL, DCAP = 5.0 pg/mL).

A.

Figure 3. Capsaicin (CAP: A) and dihydrocapsaicin (DCAP: B) plasma concentration versus time data (markers) following the nasogastric (NG) administration of cayenne pepper (n = 6, 7.5 g cayenne pepper per horse = 46.2 ± 4.4 μg/kg CAP and 25.9 ± 2.5 μg/kg DCAP). The horizontal line represents the lower limit of quantification (LLOQ) for the analytical method employed (CAP = 2.5 pg/mL, DCAP = 5.0 pg/mL). Values displayed below the LLOQ are provided for qualitative purposes only.

A.
Figure 4. Capsaicin (CAP: A) and dihydrocapsaicin (DCAP: B) plasma concentration versus time data observed (markers) and predicted (dotted lines) following topical (TOP) application of a capsaicinoid-containing ointment (n = 6, EquiBlock, CAP 31 ± 9 μg/cm² and DCAP 21 ± 7 μg/cm²). The 99.99% credibility interval is contained between the outer solid lines, and the central solid line represents the average predicted profile. The horizontal line represents the lower limit of quantification (LLOQ) for the analytical method employed (CAP = 2.5 pg/mL, DCAP = 5.0 pg/mL).
B.

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Title:
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