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Abstract

Objective To evaluate the effect of a romifidine infusion on antinociception and sedation, and to investigate its relationship to plasma concentration.

Study design Prospective, experimental, non-randomized trial.

Animals Ten healthy adult warmblood horses.

Methods Romifidine (loading dose: 0.08 mg kg^{-1}, infusion: 0.03 mg kg^{-1} $hour^{-1}$) was administered intravenously (IV) over 120 minutes. Romifidine plasma concentrations were determined by capillary electrophoresis. Sedation quality and nociceptive thresholds were evaluated at regular time points before, during and after romifidine administration. The nociceptive RIII reflex was elicited by electrical stimulation at the thoracic limb using a dedicated threshold tracking algorithm and recorded by electromyography at the deltoid muscle. A pharmacokinetic-pharmacodynamic model was established and correlation between romifidine plasma concentration and main output variables tested.

Results A two compartmental model best described the romifidine pharmacokinetic profile. The nociceptive thresholds increased compared to baseline in all horses from 10 to 146 minutes after romifidine administration ($p < 0.05$). Peak effect reached 5.7 ± 2.3 times the baseline threshold. The effect/concentration relationship followed a counter-clockwise hysteresis loop. The mean plasma concentration was weakly correlated to nociceptive thresholds ($p < 0.01$, $\rho = 0.392$). The sedative effects were significant until 160 minutes but variable, not correlated to plasma concentration ($p = 0.067$), and weakly correlated to nociceptive thresholds ($p < 0.01$, $\rho = 0.33$).

Conclusions and clinical relevance Romifidine elicited a marked antinociceptive effect. Romifidine-induced antinociception appeared with a delayed onset and lasted longer than sedation after discontinuing its administration.

Keywords antinociception, electrical stimulation, horses, nociceptive withdrawal reflex, pharmacokinetics, romifidine.

Introduction

Among sedative and analgesic drugs currently available, alpha-2 adrenergic agonists are essential for equine standing interventions. Compared to xylazine and detomidine, romifidine is more selective for the alpha-2 adrenergic receptor, evokes longer lasting sedation and tends to produce less ataxia at an equipotent sedative dose (England et al. 1992; Hamm et al. 1995; Nannarone et al. 2007). These characteristics may be advantageous for sedation during long lasting standing procedures, even though differences between alpha-2 adrenergic agonists may diminish when titrated to effect as a continuous infusion over longer time (Ringer et al. 2013).

The objective of this study was to characterize the antinociceptive effect of romifidine infusion in standing horses. Previous studies have already investigated it under experimental conditions. The hoof withdrawal latency in response to thermal stimulation (Figueiredo et al. 2005; Christovão et al. 2006) was significantly prolonged after an intravenous (IV) bolus of romifidine. The duration of effect appeared to be dose-dependent. The withdrawal latency in response to electrical stimulation increased four-fold, 15 minutes after an IV romifidine bolus (Moens et al. 2003), but less notably in response to mechanical stimulation. The nociceptive withdrawal reflex (NWR) threshold, assessed by electromyography, also significantly increased in response to single and repeated electrical stimulations after an IV romifidine bolus (Spadavecchia et al. 2005; Rohrbach et al. 2009). However, these studies only investigated specific time points after romifidine administration without correlation to plasma

concentrations, preventing a precise description of the time course (onset, duration) of antinociception elicited by romifidine, as well as comparison to its sedative properties. This limitation may explain the various durations of effect reported by the former studies, as well as the different results regarding the relationship between sedation and antinociception. Previous authors reported sedation outwearing analgesia (Lizarraga & Janovyak 2013; El-Kammar & Gad 2014), the opposite (Rohrbach et al. 2009; Costa et al. 2015), or similar time courses (Lizarraga et al. 2017).

A novel automated reflex threshold tracking system, based on a validated algorithm (von Dincklage et al. 2009), provides an opportunity to assess, nearly continuously, the NWR threshold. This methodology might allow for a more precise characterization of the time course of the antinociceptive activity, and realization of pharmacokinetic-pharmacodynamic (PKPD) modelling. The present study aimed at evaluating the antinociceptive effects of a romifidine infusion, using the automated assessment of the NWR threshold, and to investigate its relationship to plasma concentrations by creating a PKPD model. The main hypothesis was that romifidine infusion increases the NWR threshold in relation to its plasma concentration.

Material and methods

Sample size calculation

A baseline NWR threshold of 4.0 ± 0.5 mA can be expected in the horse (Spadavecchia et al. 2002; Spadavecchia et al. 2003; Spadavecchia et al. 2005; Rohrbach et al. 2009), and a two-fold increase with a standard deviation of 50% after romifidine administration would be considered relevant. Using a two-tailed paired t-test and targeting an alpha of 0.05 and a power of 95%, a sample size of 6 horses would be required (G*Power v.3.1.9.2, **, **).

A baseline sedation score of 2 (0-3) can be expected, and an increase to 5 (3-7) after romifidine administration would be arbitrarily considered relevant. Using a Wilcoxon signed rank test and targeting an alpha of 0.05 and a power of 95%, a sample size of 8 horses would

be required (G*Power v.3.1.9.2, **, **).

For a correlation (H1: $\rho > 0.85$, H0: $\rho < 0.05$) between plasma concentrations and the main output variables, targeting an alpha of 0.05 and a power of 95%, a sample size of 10 horses would be required (G*Power v.3.1.9.2, **, **).

Therefore, we chose to include 10 horses in this study. A total of nine geldings and one mare were recruited. Median age was 5 (4-16) years and mean weight was 551 ± 44 kg.

Study design

This prospective experimental study was carried as a non-randomized provocation trial to evaluate the effect of romifidine over time on the nociceptive threshold and sedation quality compared to baseline. It was approved by the Committee for Animal Experiments of **, ** (Permission number: ** **).

Animals

Ten clinically healthy warmblood horses were recruited from the National Equine Military centre. Informed consent was obtained from the centre; the horses were owned by the government. Healthy horses free from any pharmacological treatment in the two months prior to the trial were eligible for inclusion.

All horses were kept in single boxes under regular housing conditions. The experimental box was a normal stall at the same facilities. A horse not participating in the study was placed in the adjacent box. The timing of the procedures was standardized and constant environmental conditions were maintained throughout the experimental phase. Food was withheld for 12 hours prior to drug administration. In case of major complications, including cardiovascular collapse, severe ataxia with recumbency attempts and intolerance to the electrical stimulation, the horse would receive appropriate treatment and be excluded from the study.

Animal preparation

On the morning of the experiment the animal was weighed, walked into the experimental box and left undisturbed for at least 10 minutes. Physical examination was performed prior to instrumentation.

Both jugular veins were catheterized (13 gauge 105 mm catheter, Intranule; Vygon, **, **) after subcutaneous infiltration of 2 mL of Lidocaine 2% (Lidocain 2% Streuli; Streuli Pharma, **, **) The catheter on the right side was connected to a bag of Ringer's lactated solution that contained a port, in the extension set, for romifidine constant rate infusion (CRI); the left catheter was used for blood sampling.

Stimulation and recording surface electrodes were applied to the skin for NWR measurement. Skin preparation, placement site, and electrode-skin impedance were standardized. Two self-adhesive surface electrodes (Bluesensor N; Ambu, **) placed 0.5 cm apart over the left deltoid muscle, and a ground electrode (Bluesensor VL; Ambu, **) placed over the greater tubercule of the humerus were used for electromyographic (EMG) recordings (Fig. S1). In addition, two self-adhesive surface electrodes (Bluesensor N; Ambu, **) were placed 0.5 cm apart over the lateral digital nerve, between the coronary band and the metacarpophalangeal joint for electrical stimulation (Fig. S1). For each electrode, the skin was clipped, cleaned and prepared with abrader tape (Red dot Trace Prep; 3M, **), and the electrode-skin impedance was checked and kept below 2 kOhm for the duration of the experiment. If necessary, the electrode was replaced. Once instrumented, the horse was left undisturbed for 10 minutes before starting baseline measurements, and then loosely tied to the wall for the duration of the experiment.

Drug administration

After determination of the baseline NWR threshold (at least 10 minutes of a stable NWR threshold and not less than 20 minutes after starting stimulation), romifidine 0.08 mg kg^{-1} IV (Sedivet 10 mg mL^{-1}; Boehringer Ingelheim, **, **) was administered by hand over 1 minute. Immediately thereafter, the romifidine infusion (diluted to 1 mg mL^{-1} with NaCl solution) was

started at 0.03 mg kg^{-1} $hour^{-1}$ and maintained over 120 minutes using a calibrated syringe pump (Perfusor Space Syringe Pump; B. Braun, **).

NWR threshold determination

Electrical stimulations and EMG recordings were performed through a dedicated unit (Dolosys Pain Tracker; Dolosys **, **). The NWR threshold was automatically determined using a bracketing design according to the validated continual RIII reflex threshold tracking algorithm (von Dincklage et al. 2009). Each stimulation consisted of five individual rectangular pulses of a duration of 1 ms delivered at 200 Hz. The EMG activity was recorded for 500 ms with a sampling frequency of 1 kHz, starting 100 ms before the stimulation onset (noise range). The time window of interest for detecting the NWR was set between 60 ms and 200 ms after stimulation onset (NWR range). Occurrence of the NWR was defined as an interval peak Z score above 10, meaning that the difference between the maximum EMG amplitude in the NWR range and the mean EMG amplitude in the noise range had to be above the ten-fold of the standard deviation of the EMG amplitudes in the noise range (Rhudy & France 2007). Intensity of the first stimulus was set at 1 mA with a step change of 0.3 mA, increasing to 0.5 mA after three stimuli with a minimum step size of 0.3 mA. The step size increased to 0.5 mA when three changes of the stimulation intensity occurred in the same direction, and decreased back to 0.3 mA after three direction changes. The interstimulus interval was set to 10 seconds with 30% interval randomization. The measurements were automatically discarded when the EMG amplitude exceeded 15 µV in the noise range (0-100 ms before stimulation), and the stimulation intensity was repeated. Estimation of the NWR threshold is performed following every valid stimulation (not discarded due to inappropriate noise) by a logistic regression of the last 12 stimuli (von Dincklage et al. 2009).

Romifidine plasma concentrations

For determining romifidine plasma concentrations, venous blood samples were taken 10 minutes before, and 3, 5, 7, 15, 30, 55, 75, 90, 120, 150, 180 and 210 minutes after starting

romifidine infusion. For each sample, 10 mL blood was removed from the left jugular catheter and discarded, then 10 mL blood was collected into heparinized tubes and kept on ice until processing. The plasma was separated by centrifugation (10 minutes at 2000 x *g*, 10 °C), and stored in plastic cryotubes at -20 °C until analysis. Plasma concentrations were determined by capillary electrophoresis. The method used was a modification of assays previously described for the enantioselective determination of ketamine and its metabolites (Theurillat et al. 2016) and methadone and its main metabolite (Theurillat et al. 2019) in plasma. Briefly, the developed assay involves liquid/liquid extraction of romifidine and the added internal standard D-(+)-norephedrine from 100 µL of plasma using dichloromethane at alkaline pH and electrokinetic injection of the analytes (8 kV for 15 s) from the reconstituted extract across a 50 mM phosphate buffer (pH 3.0) plug. A Proteome Lab PA 800 enhanced instrument (Beckman Coulter, Fullerton, CA, USA) equipped with a 50 µm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 45 cm total length (effective length 35 cm) was used. The running buffer comprised 100 mM phosphate buffer (pH 3.0) to which 0.14 % highly sulfated γ-cyclodextrin (Beckman Coulter) was added. A voltage of 20 kV was applied and the current was about 48 µA. Sample storage and capillary cartridge temperatures were set to 18 and 16 °C, respectively. Analyte detection was achieved at 210 nm (PDA detector). Quantification of romifidine concentrations was based on five-level internal calibration using corrected peak areas. The calibration range for romifidine was 10 – 200 ng/mL and the quantification limit was 5 ng/mL. For romifidine levels of 20 and 80 ng/mL, interday precision (n=6) was 5.22 % and 2.36 %, respectively. Accuracy assessments revealed romifidine concentrations that varied less than 3 % from the target values.

PKPD modelling

For determining the romifidine pharmacokinetic profile, plasma concentrations were modelled with a commercially available software (Phoenix 64 v.8.0.0.3176 2017, WinNonLin/NLME application; Certara Inc, **, **). The most suitable mammillary

compartmental model for romifidine was determined for each individual, separately. Assessment was based on the appearance of the observed and predicted concentrations, data fit, diagnostic plots, Akaike information criterion (AIC) and residual analysis. Non-compartmental analysis was also performed to orient initial estimates. Various models were evaluated using different Non-Linear Mixed Effects algorithms. The most represented model (algorithm and number of compartments) was then applied to all the horses. Similarly, a population model was obtained considering all the collected samples.

A PKPD modelling was then performed to correlate plasma concentrations to the antinociceptive effect of romifidine. The individual NWR threshold in non-medicated horses (mean over the 5 minutes before romifidine administration) was used as baseline for each horse and the relative nociceptive threshold (divided by the individual baseline) was calculated. The effect of romifidine plasma concentrations on both the absolute and the relative nociceptive thresholds were modelled as an indirect response model:

$$dR/dt = K_{in} - K_{out} \times R$$

Where dR/dt is the rate of change of the response R (the intensity threshold required to elicit a NWR in response to the electrical stimulation) over time, K_{in} is the first-order rate constant for the factors promoting intrinsic tolerance to the noxious stimulation (increasing K_{in} will increase the threshold) and K_{out} is the zero-order rate constant for the factors increasing the nociceptive sensibility (increasing K_{out} will decrease the threshold). The fitted baseline response, R_0, is the ratio K_{in} / K_{out}. Several models were evaluated based on data fit, residual analysis and diagnostic parameters. Romifidine was found to modulate nociceptive sensibility (increase K_{out}) in a non-linear fashion (following a sigmoid Imax model) according to the following PKPD equation:

$$dR/dt = K_{in} - \{ (K_{in} / R_0) \times [1 - ((Imax_{ROM} \times C_{ROM}^{n})/(IC_{50\,ROM}+C_{ROM}^{n}) \times R)] \}$$

Where Imax is proportional to the maximal threshold (no unit), IC_{50} is the drug concentration (ng mL^{-1}) that would achieve 50% of the maximum threshold increase, and n is the slope of

the concentration-effect relationship (no unit).

The addition of an effect compartment on romifidine plasma concentration to model time delay with the observed effect was tested.

$dC_e/dt = k_{e0} \times (C_{ROM}-C_e)$

Where k_{e0} is the first order rate constant for the effect compartment, and C_e replaces C_{ROM} in the former PKPD equation.

Other pharmacodynamic parameters

The degree of sedation was scored by a multifactorial sedation scale (MFSS) ranging from 0 (no sedation) to 10 (heavily sedated) and was based on attitude, standing ability, head position, eye aperture and ear movement (Rohrbach et al. 2009). A total score of at least 5 was considered to represent effective sedation. Relative head height above the ground (HHAG) was also measured (Ringer et al. 2012). Sedation was assessed at selected time points (baseline, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 125, 130, 135, 140, 145, 150, 160, 170, 180, 190, 200 and 210 minutes).

Heart rate, respiratory rate and gut motility were evaluated at regular intervals after sedation assessment. Adverse effects were registered throughout the experiment as well as frequency of urination, defecation and behavioural reactions.

Statistical analysis

Statistical analyses were carried out using SigmaPlot for Windows (SigmaPlot v.14; Systat Software GmbH, **, **). Data were tested for normal distribution by visual inspection and confirmed by a Shapiro-Wilk test. Normally distributed data are presented as mean ± SD for values from a sample, or mean [95% confidence interval] for values from a population. Non-normally distributed data are presented as median (range). The effect of treatment on NWR thresholds and HHAG was tested using one-way ANOVA for repeated measures. The effect of treatment on sedation scores was tested using Friedman ANOVA on ranks for repeated measures. Post-hoc pairwise multiple comparisons were performed with a Tukey test. Linear

231 correlation between plasma concentrations, NWR thresholds, and sedation scores were tested
232 with Pearson product moment analysis.
233
234 **Results**
235 All horses completed the study.
236 *Pharmacokinetic profile*
237 A mean steady plasma concentration for romifidine of 28.3 [24.4-32.2] ng mL^{-1} was
238 maintained from 30 to 120 minutes after start of the infusion (Fig. 1). A two compartmental
239 model best described romifidine pharmacokinetic profile (Table S1).
240 *Antinociceptive effect and PKPD modelling*
241 The mean baseline NWR threshold before romifidine administration was 4.6 ± 1.7 mA (Table
242 1). The NWR threshold increased ($p < 0.001$) compared to baseline in all horses from 10 to
243 146 minutes after romifidine administration ($p < 0.05$), up to a peak value of 5.7 ± 2.3 times
244 the baseline. The onset for the antinociceptive effect of romifidine, arbitrarily defined as the
245 time to reach 75% of the maximal effect for each horse, was 22.2 ± 6.9 minutes and the offset
246 of the antinociceptive effect, arbitrarily defined as the time to decrease to 25% of the maximal
247 effect for each horse once the infusion was stopped, was 36.7 ± 14.6 minutes. When
248 romifidine reached steady plasma concentrations (30 to 120 minutes after infusion start), the
249 NWR threshold was 4.3 [3.1-5.5] times above the baseline (Fig. 2). One horse (identified
250 number 4) appeared as an outlier increasing its NWR threshold 9.5 ± 0.7 times above its
251 baseline. Among the other horses, the NWR threshold during this time period was 3.8 [3.2-
252 4.4] times above the baseline.
253 The relative NWR threshold appeared to represent better romifidine antinociception than the
254 absolute threshold which exhibited more individual variability and a larger confidence
255 interval. It was more significantly correlated to romifidine plasma concentrations and the
256 PKPD models were more accurate (Fig. 3). Therefore, results for PKPD modelling are

257 presented only for the relative threshold. An indirect sigmoid Emax response model was
258 obtained for each horse as well as for the population, with k_{e0}, K_{in}, EC_{50} and n as PD
259 parameters including random effect (Emax = 1, K_{out} = 0.9-1.1*K_{in}). The best population
260 estimates were 0.46 $minute^{-1}$, 0.27 $minute^{-1}$, 10.9 ng mL^{-1}, and 1.07, respectively (Table S2).
261 The graphical representation of the relative NWR threshold over the plasma concentration
262 (from the population PKPD model) followed a counter-clockwise hysteresis loop (Fig. S2).
263 The individual relative NWR thresholds were not well correlated (p = 0.19, ρ = 0.19) to their
264 respective romifidine plasma concentrations (Fig. 4). This was supported by individual
265 variability of IC_{50} (9.66 ± 3.49 ng mL^{-1}). Still, a weak linear correlation (Pearson Product
266 Moment, ρ = 0.392) was significant (p = 0.007) when the horse identified as number 4
267 (outlying high NWR threshold) was excluded from analysis.
268 *Other pharmacodynamic effects*
269 The median sedation score reached its maximal value of 7 (5-9) at the first evaluation (5
270 minutes after infusion start). The individual sedation scores reached their maximal value of 8
271 (6-9) at 20 (5-70) minutes after infusion start. All the horses reached effective sedation
272 (MFSS ≥ 5) within 20 minutes after the start of the infusion. The sedation score was 6 (2-9)
273 during steady plasma concentrations of romifidine (from 30 to 120 minutes after infusion
274 start). The sedation scores were different from baseline (p < 0.05) up to 160 minutes after the
275 infusion start. The sedation offset (score < 5) occurred before the end of the infusion in 5
276 horses (identified as numbers 2, 4, 5, 8, 9) and 5 (0-25) minutes after termination of the
277 infusion in the five other horses. The sedation scores obtained during romifidine infusion
278 were not correlated with plasma concentrations (Pearson Product Moment, ρ = 0.26, p =
279 0.067). However, sedation scores exhibited a weak linear correlation with their respective
280 mean NWR thresholds (Pearson Product Moment, ρ = 0.33, p < 0.001). During steady plasma
281 concentrations of romifidine (from 30 to 120 minutes after infusion start) the relative HHAG
282 was 50 [45-55] % exhibiting large variability and appeared to not adequately reflect sedation

quality, thus results are not reported.

No major adverse effects were observed during the study. All horses tolerated the nociceptive stimulations well, without exaggerated behavioural reactions. The romifidine caused increased urination in all the horses. One horse (identified as number 10) exhibited severe ataxia without attempts to be recumbent.

Discussion

In the present study, administration of an intravenous romifidine infusion in standing horses led to a significant increase in the NWR threshold (about 4 times its baseline), supporting an antinociceptive effect of romifidine. The relevant antinociceptive effect lasted from approximately 20 minutes after the start of the infusion until approximately 35 minutes after the end of infusion. The amplitude of the NWR threshold increase was weakly correlated to individual romifidine plasma concentration. Sedation quality and duration did not correlate with the antinociceptive effects. At the dose regimen administered, the sedation became insufficient (MFSS <5) before the end of romifidine infusion in half of the horses.

The nociceptive threshold measured in the present study was in agreement with former studies. Baseline values measured before treatment administration were in accordance with previous reports applying electrical stimulation at the thoracic limb in conscious, non-medicated horses (Spadavecchia et al. 2002; Spadavecchia et al. 2003; Spadavecchia et al. 2005; Rohrbach et al. 2009). Romifidine increased the nociceptive threshold to 5.8 times its baseline after a single bolus of 0.08 mg kg^{-1} (Rohrbach et al. 2009). Another publication reported a milder effect of romifidine on the nociceptive threshold (an increase to only 3 times its baseline), however a different method of determination as well as different time points (at 5 and 25 minutes after administration) were used (Spadavecchia et al. 2005). In the present study, the romifidine infusion (0.03 mg kg^{-1} $hour^{-1}$) maintained a nearly constant nociceptive threshold.

As in previous reports, the nociceptive threshold increased already a few minutes after romifidine administration (Spadavecchia et al. 2005; Rohrbach et al. 2009). However, past reports were limited to measurements at few time points with large intervals. The methodology used here characterizes continuously the effect of the drug allowing for a precise determination of threshold changes. In the present study, the nociceptive threshold reached a significant difference from baseline values 10 minutes after administration. The onset of maximal antinociception (defined as reaching 75% of the peak nociceptive threshold) was about 20 minutes after the start of romifidine administration. After termination of the infusion, in the present study, the threshold decreased down to 25% of the peak value within 35 minutes and was not significantly different from baseline anymore already at 26 minutes. This is markedly different than the 55 or 120 minutes reported in previous publications after IV bolus (Spadavecchia et al. 2005; Rohrbach et al. 2009). This difference may be the result of methodological diversity including different administration regimen as well as individual variability for termination of the effect.

Although the nociceptive threshold increased and decreased rapidly after starting and terminating the romifidine infusion, the relationship between the observed effect and the concentration time course in blood obtained from the PKPD model revealed a marked hysteresis. Counter-clockwise hysteresis, together with the prolonged termination of the effect, support a time delay due to a possible longer equilibration time between plasma and the site of action or a mechanistic response delay (Fan & de Lannoy 2014). A similar delay has been previously observed for the sedative effects of alpha-2 agonists, and is reported to be more pronounced with romifidine than xylazine or detomidine (Wojtasiak-Wypart et al. 2012, Ringer et al. 2013).

The sedative effect of romifidine administered as a single IV bolus (0.08-0.1 mg kg^{-1}) has been reported to correlate to the drug plasma concentration (Wojtasiak-Wypart et al. 2012; de Vries et al. 2016; Cenani et al. 2017; Romagnoli et al. 2017). In the present study, sedation

appeared within 5 minutes after romifidine administration and vanished very rapidly after discontinuation. This is less than the 60 minutes of recovery reported in a previous study (Ringer et al. 2012), even though different assessment endpoints were applied. Moreover, half of the horses did not maintain satisfactory sedation during romifidine infusion, while the same dosage had been proven satisfactory in other studies (Ringer et al. 2012; Ringer et al. 2013). Interestingly, similar romifidine plasma concentrations close to 30 ng mL^{-1} were observed (Ringer et al. 2012). The setting, and in particular whether the horses were stimulated, manipulated or received painful interventions, will very probably influence the relationship between quality of sedation and antinociceptive intensity. The plasma concentrations were similar, but nociceptive stimulation was not elicited in the latter investigations. When applying the same dose regimen in horses undergoing dental procedures, 5 out of 11 required additional romifidine boli (Marly et al. 2014).

Beside the sedation being more variable and more difficult to quantify, its time course differed markedly from antinociception. There are controversial results on this point in the literature (Valverde 2010), probably in part due to difficulty to evaluate depth of sedation. Data obtained in the present study suggests that sedation both appears and stops earlier than antinociceptive effects. This is in agreement with some of the previous publications (Rohrbach et al. 2009; Costa et al. 2015).

Applying different evaluation systems to quantify depth of sedation probably contributes to these discrepancies, and difficulties to provide a reliable index of sedation have been reported (Schauvliege et al. 2019). Many of the former studies reported HHAG to be an adequate method to quantify sedation in horses (England et al. 1992; Hamm et al. 1995; Freeman & England 2000; Figueiredo et al. 2005; Ringer et al. 2012; Ringer et al. 2013). In the present study, the results obtained using HHAG appeared largely variable and poorly correlated to sedation. This was probably the result of our experimental setting, where horses were tied up, continuously stimulated, subject to clinical examinations and blood was regularly sampled.

Other limitations of the current study should be taken into consideration. It is not clear in which extent the NWR threshold accurately reflects a clinical level of pain perceived by the horses, and the threshold value cannot be directly transposed to a clinical situation. However, NWR threshold has been associated with pain threshold in humans (Willer 1977), and is used extensively as a non-invasive and objective method to study nociception and its pharmacological modulation (Willer & Bathien 1977). The methodology has also been validated for assessment of nociception in horses (Spadavecchia et al. 2002; Luna et al. 2015; Spadavecchia et al. 2016). For instance, the amplitude of the EMG-derived NWR at low stimulation intensity (at threshold level) has been correlated with an active behavioural reaction (limb withdrawal) in response to painful stimulations (Spadavecchia et al. 2002; Spadavecchia et al. 2003).

This study has been performed in healthy, experimental animals; therefore caution should be taken while extrapolating the results to horses under clinical conditions. Moreover, sex distribution was uneven. Sex differences in pain and antinociception have been reported in humans and animal models (Greenspan et al. 2007) therefore, these results should be extrapolated carefully to the general equine population. Another potential limitation is the lack of a control group. In a non-randomized provocation trial, the effect of treatment cannot be distinguished from the effect of time. Depending on the stimulation paradigm used, habituation or sensitization to the nociceptive stimulation may happen (Arendt-Nielsen et al. 2000; von Dincklage et al. 2013). Previous validation trials of the experimental setting applied in the present study showed that the NWR is expected to remain constant over time (von Dincklage et al. 2009). Finally, the observer was aware of the treatment. Observer bias has been reported when using subjective measurement scales (Hrobjartsson et al. 2013). This may apply for the evaluation of sedation, but determination of the NWR threshold was performed automatically by the pain tracker unit and was not expected to be influenced by the observer.

In conclusion, the present study confirms the marked antinociceptive effect of romifidine.

Furthermore, the study provides a precise characterization of its time course when administered as an infusion regimen. Compared to sedation, romifidine antinociception appeared with a delayed onset and lasted longer after discontinuing its administration.

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Table 1 Absolute values of the nociceptive withdrawal reflex threshold (NWRT) measured during intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses. The baseline values (±SD) were measured before treatment. The peak values were the maximal NWRT (±SD) measured during romifidine administration. The mean values were the average NWRT [95% confidence interval] measured between 30 and 120 minutes after start of the infusion.

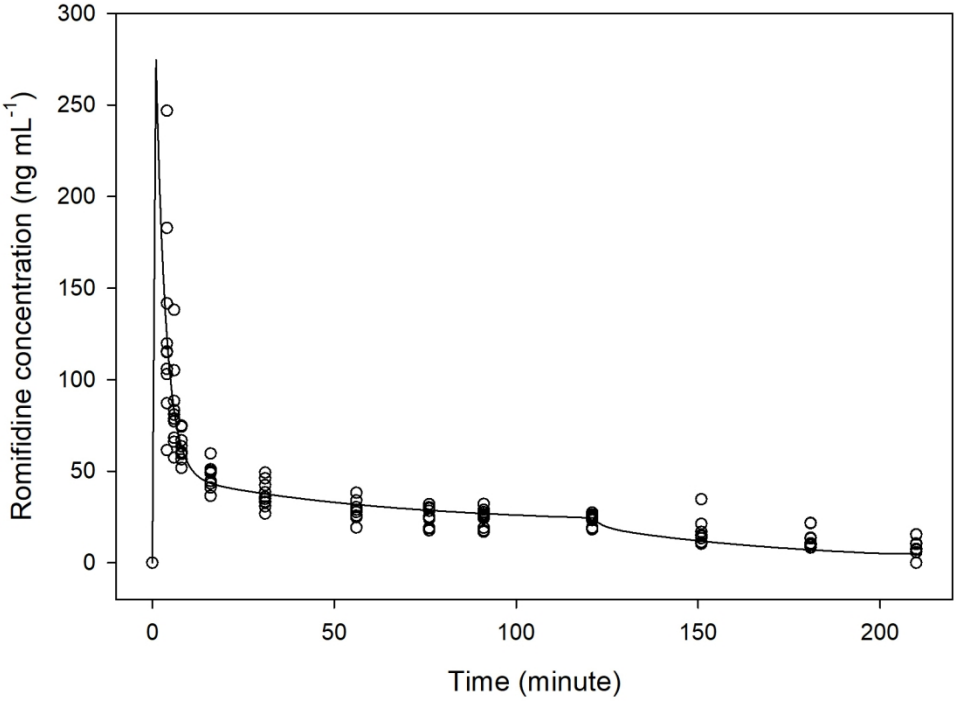
Parameters	Horse identification number											
	1	2	3	4	5	6	7	8	9	10	Mean	SD/CI
Baseline (mA)	2.4	4.0	3.4	6.1	2.8	5.9	5.9	3.3	4.7	7.5	4.6	1.7
Peak value (mA)	15.7	18.3	19.6	69.1	16.2	36.5	19.5	10.9	26.8	37.3	27.0	17.2
Mean value (mA)	10.4	14.1	14.9	58.1	13.0	25.7	15.6	8.7	17.9	29.5	20.8	11.8-29.8

Table S1 Individual and population (Pp) pharmacokinetic parameters for romifidine administered as an intravenous infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses.

	1	2	3	4	5	6	7	8	9	10	Pp
V_1 (mL kg^{-1})	349.42	774.80	702.48	171.46	663.96	577.89	1006.58	146.74	495.43	856.16	250.32
V_2 (mL kg^{-1})	886.8	1709.6	892.8	1306.3	1221.3	1848.6	3264.1	1495.8	1031.9	1469.1	842.6
k_{10} ($minute^{-1}$)	0.07	0.04	0.03	0.11	0.03	0.04	0.02	0.09	0.05	0.01	0.10
k_{12} ($minute^{-1}$)	0.10	0.04	0.07	0.21	0.03	0.05	0.03	0.19	0.09	0.03	0.22
k_{21} ($minute^{-1}$)	0.04	0.02	0.06	0.03	0.02	0.01	0.01	0.02	0.04	0.02	0.07

Table S2 Individual and population (Pp) pharmacokinetic-pharmacodynamic parameters for the effect of romifidine administered as an intravenous infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) on the relative nociceptive withdrawal reflex threshold in ten experimental horses.

	1	2	3	4	5	6	7	8	9	10	Pp
K_{in} ($minute^{-1}$)	0.59	0.17	0.29	0.26	0.25	0.20	0.25	0.24	0.27	0.24	0.27
K_{out} ($minute^{-1}$)	0.58	0.19	0.29	0.28	0.27	0.20	0.25	0.24	0.29	0.26	0.25
k_e0 ($minute^{-1}$)	0.11	0.95	0.60	0.51	1.00	0.50	0.66	0.51	0.97	1.00	0.46
IC_{50} (ng mL^{-1})	9.27	12.87	6.90	7.84	7.52	10.00	6.02	13.77	6.29	16.12	10.93
n	0.99	2.06	0.90	1.00	0.97	1.00	0.96	1.35	0.87	1.62	1.07



Individual plasma concentrations of romifidine (ng mL-1) (red dots) and predicted concentration from the population pharmacokinetic model (straight line) during and after intravenous romifidine infusion (0.08 mg kg-1 followed by 0.03 mg kg-1 hour-1 for 120 minutes) in ten experimental horses.

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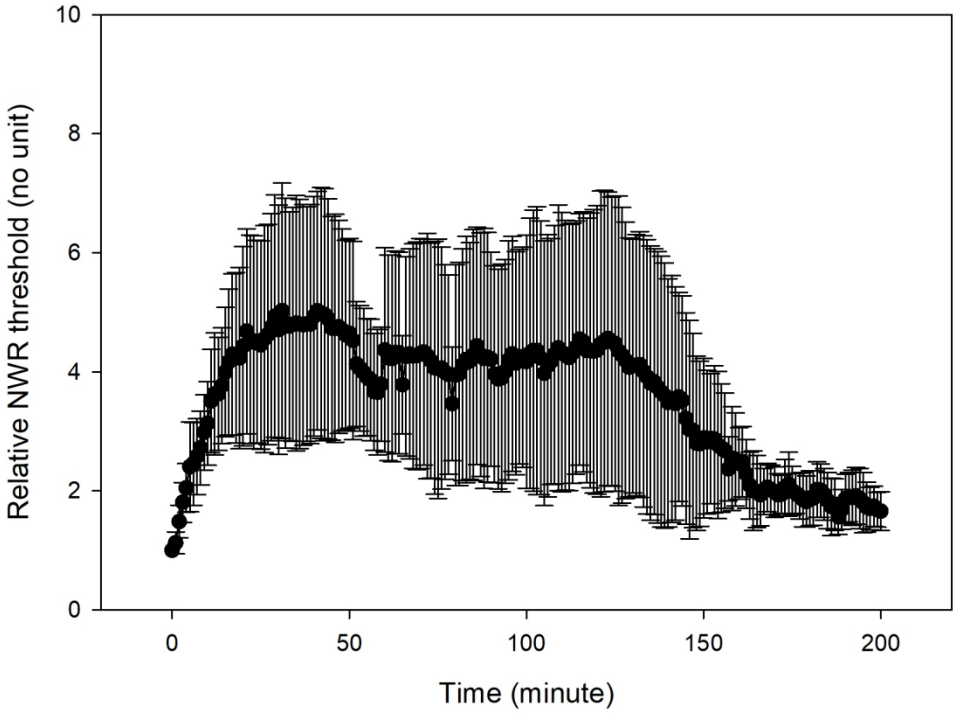

Mean (±SD) nociceptive withdrawal reflex threshold relative to individual baseline values during and after intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses.

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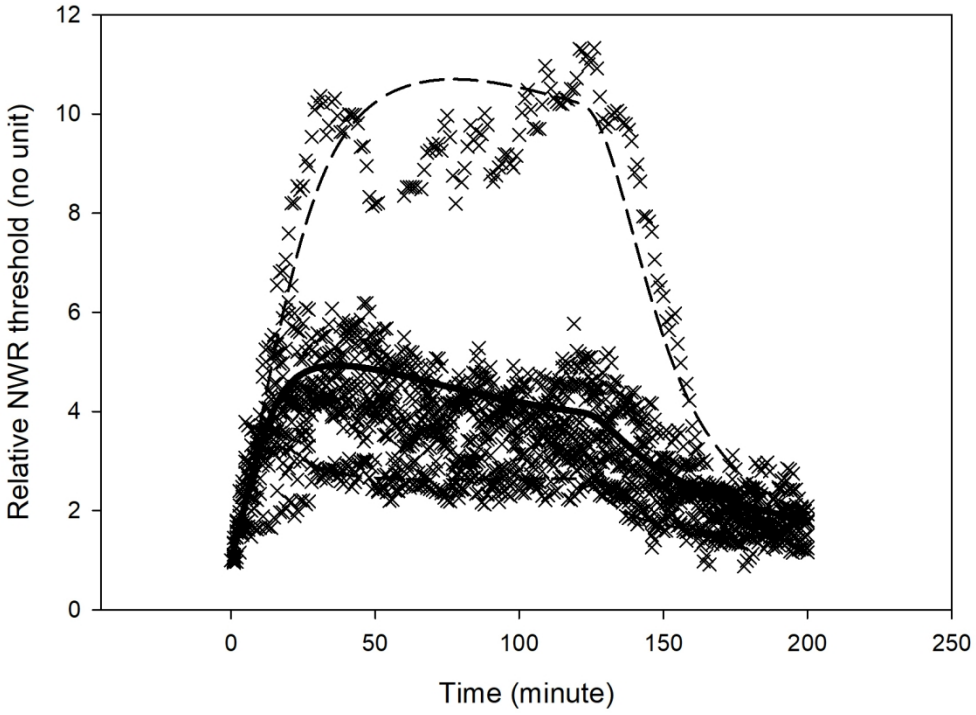

Individual relative nociceptive withdrawal reflex (NWR) thresholds (thin crosses) during and after intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses, as well as the predicted time-course of the NWR threshold (PK/PD model) for the population (straight line) and two out of the ten horses (green and red dashed lines).

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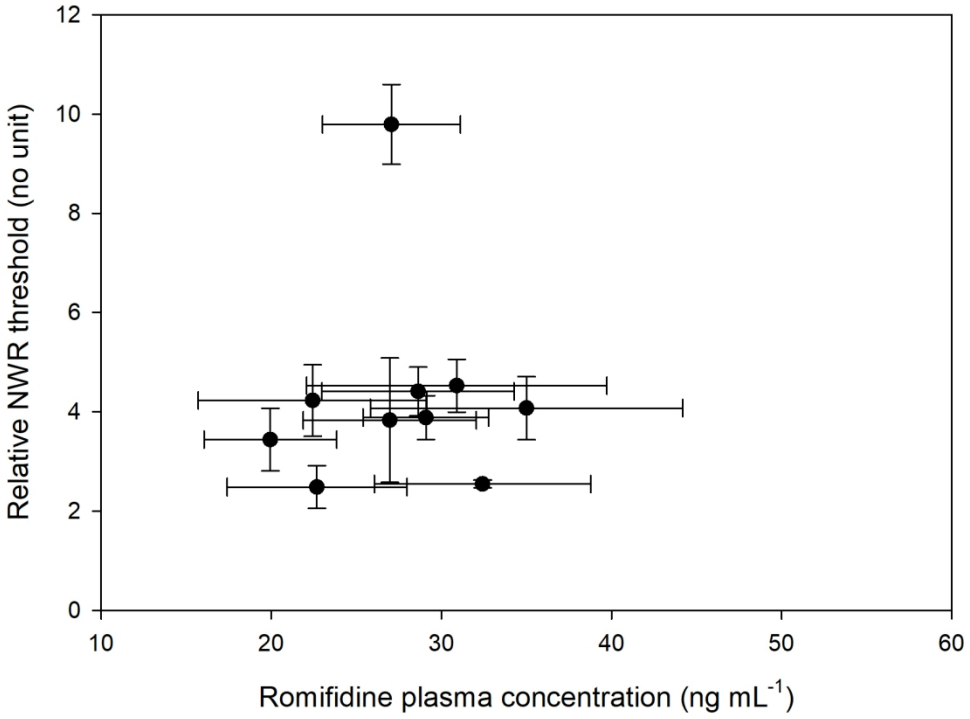

Individual means (±SD) of the relative nociceptive withdrawal reflex thresholds against their corresponding mean (±SD) plasma concentration during intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses. Data are calculated from time points 30, 55, 75, 90, and 120 minutes after the start of the romifidine infusion.

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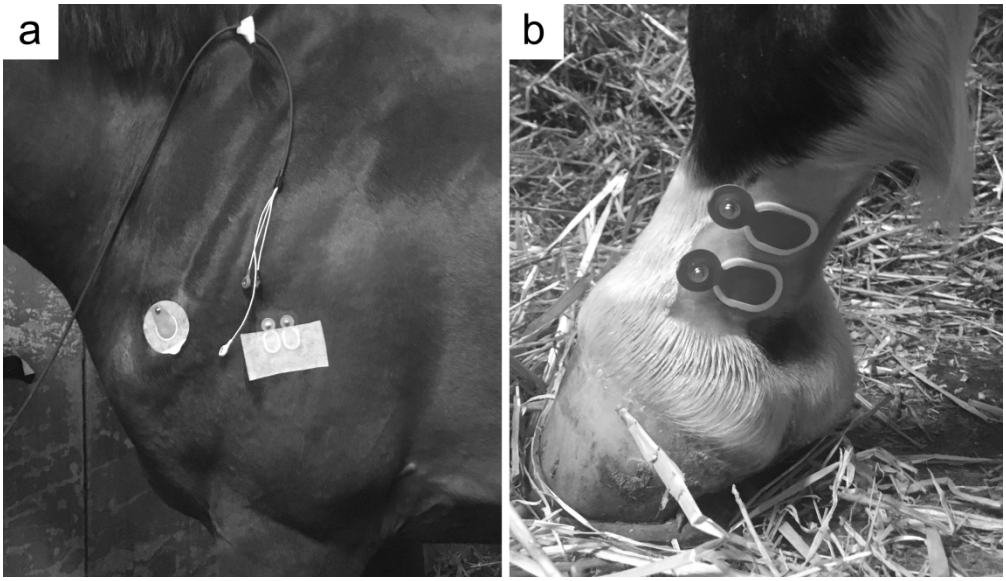

Placement of recording (a) and stimulating (b) electrodes for measurement of the nociceptive withdrawal reflex prior to and during a continuous intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes).

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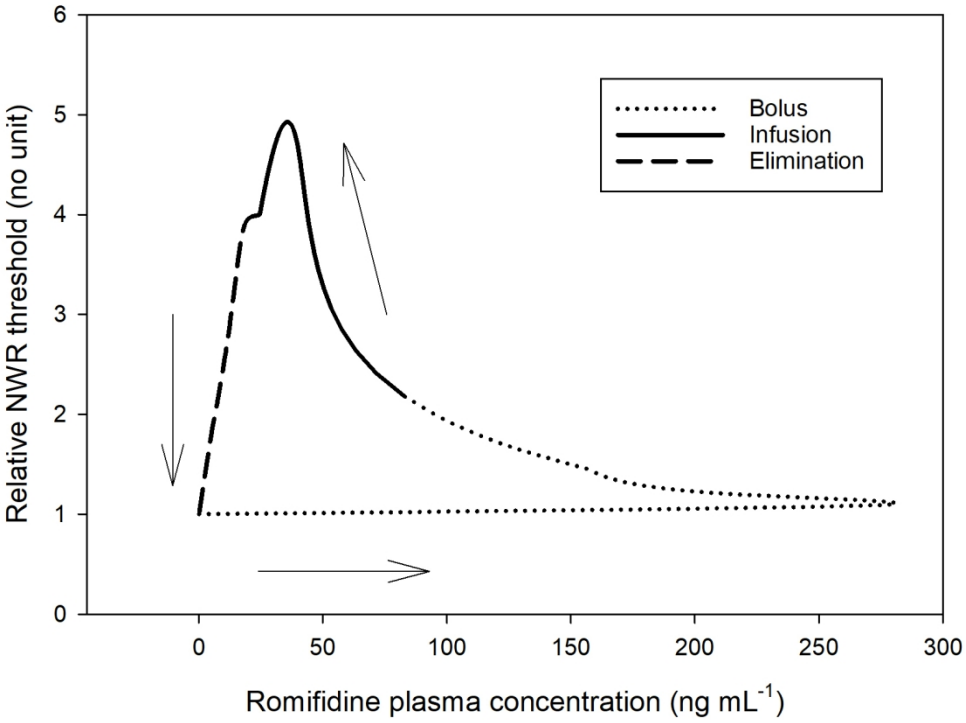

Counter-clockwise hysteresis loop of the relationship between effect (nociceptive withdrawal reflex threshold increase) and plasma concentration during intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) based on a population pharmacokinetic-pharmacodynamic model from ten experimental horses.

148x118mm (300 x 300 DPI)

Figure legends

Figure 1 Individual plasma concentrations of romifidine (ng mL^{-1}) (red dots) and predicted concentration from the population pharmacokinetic model (straight line) during and after intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses.

Figure 2 Mean (±SD) nociceptive withdrawal reflex threshold relative to individual baseline values during and after intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses.

Figure 3 Individual relative nociceptive withdrawal reflex (NWR) thresholds (thin crosses) during and after intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses, as well as the predicted time-course of the NWR threshold (PK/PD model) for the population (straight line) and two out of the ten horses (green and red dashed lines).

Figure 4 Individual means (±SD) of the relative nociceptive withdrawal reflex thresholds against their corresponding mean (±SD) plasma concentration during intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) in ten experimental horses. Data are calculated from time points 30, 55, 75, 90, and 120 minutes after the start of the romifidine infusion.

Figure S1 Placement of recording (a) and stimulating (b) electrodes for measurement of the nociceptive withdrawal reflex prior to and during a continuous intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes).

Figure S2 Counter-clockwise hysteresis loop of the relationship between effect (nociceptive withdrawal reflex threshold increase) and plasma concentration during intravenous romifidine infusion (0.08 mg kg^{-1} followed by 0.03 mg kg^{-1} $hour^{-1}$ for 120 minutes) based on a population pharmacokinetic-pharmacodynamic model from ten experimental horses.