Sveriges lantbruksuniversitet
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Uppsala

2013

Examensarbete inom veterinärprogrammet

ISSN 1652-8697
Examensarbete 2013:19

SLU
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Plasmakortisolhalter efter behandling med enbart metadon eller tillsammans med acepromazin eller detomidin hos hästar

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Examensarbete inom veterinärprogrammet, Uppsala 2013
Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för biomedicin och veterinär folkhälsovetenskap
Kurskod: EX0751, Nivå A2E, 30hp

Key words: methadone, cortisol, acepromazine, detomidine, horse
Nyckelord: metadon, kortisol, acepromazin, detomidin, häst

Online publication of this work: http://epsilon.slu.se
ISSN 1652-8697
Examensarbete 2013:19

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SUMMARY

In small animals morphine-like drugs, opioids, are often used as analgesia and to reduce the use of anaesthetic drugs. Opioids have also been administered to horses for more than 70 years by practicing veterinarians but the use has been limited and controversial. This is because horses are very sensitive to all the adverse effects from opioids, like excitation. Methadone is a μ-receptor agonist, the receptor mainly responsible for the opioids analgesic effects. Methadone is not approved for the use in animals in Sweden but is still used *off-label* frequently because of its good pain relieving effect. Cortisol is a blood parameter often used to objectively measure stress, pain and analgesia in animals. In other animals than horses, plasma cortisol levels are affected by administration of synthetic opioids, like methadone, but how the cortisol release is affected depends on what opioid is used, the treatment protocol performed and the species tested.

The aim of this study was to examine how methadone *per se*, either alone or together with acepromazine or detomidine, affects the plasma cortisol concentrations in horses.

The study design used was a randomized, placebo controlled and blinded study with a *cross-over* design. The horses studied were eight adult warm-blood trotters, six mares and two geldings. The treatment protocols were; methadone (0.2 mg/kg i.v.) together with saline intramuscularly (i.m.), methadone (0.1 mg/kg i.v.) with acepromazine (0.05 mg/kg i.m.), methadone (0.1 mg/kg i.v.) with detomidine (0.01 mg/kg i.m.) and saline i.v. and i.m. as a control. There was at least a one week *wash out*-period between the treatments.

Plasma cortisol was measured using a commercial cortisol ELISA kit. A validation of this method for the use in horses was done. The method worked well on horse plasma in the concentration range between 20-800 ng/mL. The within and between assay variability of this study were also very low which indicated that the concentrations obtained were quite accurate.

After administration of methadone there was a statistical significant rise in the plasma cortisol concentration compared to the control value ($p < 0.05$). There was also a significant rise in the plasma cortisol concentration after administration of methadone + detomidine ($p < 0.05$) but no significance was seen after administration of methadone + acepromazine.

The results of this study showed that i.v. administered methadone *per se*, either alone or together with detomidine, induced the release of plasma cortisol in horses. To what extent methadone affects the plasma cortisol concentrations seems to be dependent on which substance methadone is used together with. These results indicate that the plasma cortisol concentration may not be a useful parameter for stress or pain when evaluating analgesia of methadone in horses.

SAMMANFATTNING

Opioider är vitt använt på smådjur som smärtlindring och för att minska behovet av anestesimedel. Opioider har också nyttjats på hästar så långt som 70 år tillbaka i tiden men användandet har varit begränsat och kontroversiellt då hästar lätt exciterar på grund av opioider. Metadon är en μ-receptoragonist och detta är den receptor som främst står för opioidernas analgetiska effekt. Metadon är inte godkänd för användning på djur i Sverige men används ändå ofta *off-label* på grund av sin goda smärtlindrande förmåga. Kortisolhalten i blodet är en parameter som ofta används för att objektivt utvärdera stress, smärta och smärtlindring hos djur. Hos andra djurslag än häst påverkas kortisolfrisättningen av opioidgivor men exakt hur kortisolfrisättningen i blodet påverkas beror på val av opioid, behandlingsprotokoll samt djurslag.

Syftet med den här studien var att undersöka hur metadon *per se*, antingen självt eller tillsammans med acepromazin eller detomidin, påverkar kortisolkoncentrationen i blodet hos hästar.

Studien var utformad med en *cross-over* design samt var randomiserad, placebokontrollerad och blindad. Hästarna som ingick i försöket var åtta stycken vuxna varmblodstravare, sex ston och två vallacker. De behandlingsprotokoll som undersöktes var; metadon (0,2 mg/kg i.v.) tillsammans med natriumklorid (NaCl) i.m., metadon (0,1 mg/kg i.v.) tillsammans med acepromazin (0,05 mg/kg i.m.), metadon (0,1 mg/kg i.v.) tillsammans med detomidin (0,05 mg/kg i.m.) och NaCl i.v. och i.m. som kontroll. Det var en veckas *wash-out*-period mellan försöksomgångarna.

Plasmakortisolhalter undersöktes med ett kommersiellt ELISA kit och en validering för häst utfördes. Metoden fungerade bra på hästplasma med kortisolhalter mellan 20-800 ng/ml. Variationskoeffiecienten, både inter-och intra-test, var låg i den här studien vilket indikerar ett korrekt uppnått resultat.

Efter metadongivan var det en statistisk signifikant ökning av kortisolkoncentrationen i plasman jämfört med kontrollen ($p < 0,05$). En signifikant skillnad med förhöjt kortisol, jämfört med kontrollen, sågs också efter metadon+detomidin-giva ($p < 0,05$) men inte efter metadon+acepromazin-giva.

Resultaten i denna studie visade att intravenös metadongiva, som enda läkemedel eller tillsammans med detomidin, ger en frisättning av kortisol till blodet hos hästar. Till vilken grad kortisolfrisättningen uppstår beror på vilket läkemedel metadon ges tillsammans med. Dessa resultat visar att plasmakortisolhalten troligen inte är en bra parameter att studera när metadons analgetiska egenskaper ska studeras och utvärderas hos hästar.

INTRODUCTION

Pain assessment and pain treatment in humans and small animals are areas that have been studied quite extensively but for horses there is still a lot of research yet to be done. The horse today has gone from being a work tool to being more of a companion animal and therefore more research about pain treatment in horses is requested (Taylor, Pascoe & Mama, 2002).

In small animals morphine-like drugs, opioids, are often used pre-, peri, and post-operative to minimize pain and to reduce the use of anaesthetic drugs. Opioids have also been administered to horses for more than 70 years by practicing veterinarians but the use in horses is not as simple as in small animals. This is because there is a small window between pain relief and excitation and therefore horses are more prone to side effects from the opioids than small animals (Bennett & Steffey, 2002).

Methadone is a μ-receptor agonist, the most important receptor responsible for the opioids analgesic effects. Methadone is not approved for the use in animals in Sweden but is used *off-label* frequently.

To evaluate the analgesic effect of a drug different parameters in the blood are often used as indicators of stress and pain. The cortisol concentration in the blood is an example of an indicator often used for stress and pain evaluation in animals (Ayala *et al*, 2012; Mircean *et al*, 2007). Opioids themselves have been shown to affect the cortisol concentration in the blood by stimulating or inhibiting the release of hormones from the hypothalamus, pituitary gland or adrenal glands (Pechnick, 1993). If the cortisol release is stimulated or inhibited varies and depends on which opioid is used, how the experiment is designed and what species is tested. It has been shown in cats that the cortisol concentration rises after a single dose of morphine (Borrell, Llorens & Borrell, 1975) whereas a single dose of morphine lowered the cortisol concentration in the blood in cattle (Nanda, Dobson & Ward, 1992). Goats also respond with a plasma cortisol rise after a single dose of buprenorphine but the response is different when tested three weeks later (Ingvast-Larsson *et al*, 2007).

The cortisol concentration in the blood varies with a lot of different factors and this is important to consider when evaluating the results. In horses the release of cortisol follows both a circadian and an episodic rhythm with high levels early in the day to very low levels in the afternoon and night (Toutain *et al*, 1988). The concentration also varies with age (Fazio *et al*, 2009) and alters easily with changes in the environment and handling of the horse (Irvine & Alexander, 1994). To get a sample as correct as possible it is important to think about all of these factors when analysing cortisol in horses (Hart, 2012).

This study is part of a larger research project with the aim of investigating the use of methadone for horses. Part of the larger research project is to investigate how methadone affects some parameters used for pain assessment. Cortisol is a common parameter in pain assessment. How methadone affects the cortisol release in horses is unknown, therefore a study like this is important to do for better understanding of pain and how to assess analgesia in horses. The aim of this study is to examine how methadone *per se* affects the cortisol levels in the blood in horses. Methadone is often used in a combination with other drugs when

administered to horses therefore these combinations will also be examined as for the release of cortisol in the blood. The combinations that will be examined are methadone, methadone + acepromazine, methadone + detomidine (an α_2-agonist) and placebo (NaCl).

BACKGROUND

Pharmaceuticals

Methadone

There are three main opioid receptors, μ, δ and κ and the μ-receptor is the most important receptor responsible for the opioids analgesic effects (Rang *et al*, 2007). Methadone is a μ-receptor agonist (Mason, 2004). All the receptors are G-protein linked and are distributed in the brain, spinal cord and also in other tissues like synovial membranes (Mason, 2004). Methadone, in contrast to morphine, is also an NMDA (N-methyl-D-aspartate) receptor antagonist. When the NMDA receptor is activated central sensitization can occur because of lowered pain threshold in the central nervous system. Blocking of the receptor can reduce rapid tolerance development, otherwise common during opioid treatment (Davis & Walsh, 2001). Methadone also inhibits the re-uptake of serotonin and norepinephrine in the brain which can lead to a better analgesia (Davis & Walsh, 2001).

The dose normally recommended for horses is 0.1 mg/kg i.v. or i.m. but the recommended dose can vary widely and higher doses are also often used (Clutton, 2010). Methadone in horses, after i.v. administration, has shown to have a quite short elimination half-life (t½). In one study the t½ was approximately 1 h (Linardi *et al*, 2012) and in another it was about 1.34 h (Wellme Karlsson, 2011) after i.v. administration. Horses are also prone to excitation due to opioid administration. The reason for this is that there is a narrow margin between analgesia and excitation. Pain-free horses treated with opioids seem to be more susceptible to excitation than horses in pain (Robertson & Sanchez, 2010).

Acepromazine

Acepromazine acts as an antagonist of the dopamine D_2 receptors. The drug has a general calming effect but the horse tend to stay reactive to noise and other stimulation after a dose of acepromazine (Mason, 2004). There can be quite some side effects because acepromazine is not only antidopaminergic but for example also acts as an antagonist at α_1-adrenoreceptors, histamine-H1 receptors and serotonin receptors (Rang *et al*, 2007). Examples of side effects are lowered haematocrit, decreased blood pressure, priapism and lowered seizure threshold. Acepromazine is nevertheless often used in horses in combination with an opioid or an α_2-agonist for pain relief, sedation for minor procedures or as a preanesthetic drug (Mason, 2004). It is important to remember that acepromazine itself does not induce analgesia. The drug got a long duration of effect with maximum plasma levels after 30 min after i.m. administration and the t½ is 2-3 h (Mason, 2004).

Detomidine

This group of drugs act through binding to α_2-adrenoreceptors. These receptors can be found in the brain, spinal cord and in nerve terminals throughout the body. The main function of the receptors is to inhibit the release of the neurotransmitter norepinephrine. The receptors in the

CNS mediate sedation, analgesia and decreased sympathetic nervous system output and the receptors more peripherally located mediate vasoconstriction and diminished insulin release (Rang *et al*, 2007). α_2-agonists are very commonly used in horses for sedation, pain relief in for example colic and to reduce the use of inhalation anaesthetics. When given i.v. a phase of hypertension first develops followed by a mild hypotension. By the same time a decrease in heart rate occurs and this reduces the cardiac output. The drugs also relax the smooth muscles in the airways and can decrease the gastrointestinal motility (Mason, 2004). The onset of sedation after i.v. administration is fast and peak plasma concentrations occur about 12-15 min after i.m. administration. The elimination half-life is approximately 50-75 min after i.v. administration (50-75 min for xylazine, 70 min for detomidine and 50 min for medetomidine) (Mason, 2004).

Cortisol

General aspects

Cortisol is a glucocorticoid secreted from the cortex of the adrenal glands. The secretion is regulated by ACTH (adrenocorticotropic hormone) from the anterior pituitary that, in turn, is regulated by CRH (corticotropin-releasing hormone) from the hypothalamus. This is called the hypothalamic-pituitary-adrenal (HPA) axis. The cortisol also affects the pituitary and hypothalamus by actions of negative feedback. Cortisol is synthesised from cholesterol and is transported in the blood bound to proteins, CBG (corticosteroid-binding globulin) (Sjaastad, Hove & Sand, 2003). The cortisol plasma half-life in horses varies, due to a nonlinear binding to CBG, but can be up to 1,55 ± 0,33 h (Lassourd *et al*, 1996). Cortisol is, in most diurnal species, secreted with a circadian variation, most common with high concentrations at the end of night and in early morning and with low concentrations in the afternoon and night (Sjaastad, Hove & Sand, 2003). The opposite applies to nocturnal species like the rat, which mainly secretes another hormone called corticosterone instead of cortisol, with high levels during the evening and night and low levels in the morning and day (Atkinson & Waddell, 1997).

Cortisol is an important stress hormone and facilitates the action of other hormones such as epinephrine and glucagon. The main action of cortisol is to increase plasma concentrations of glucose, fatty acids and amino acids, exert an anti-inflammatory effect and to suppress the immune system. This happens during stress because cortisol stimulates gluconeogenesis, glycogenolysis, inhibit uptake of glucose in the tissues and stimulates degradation of fat and proteins. During long periods of stress cortisol exerts a growth-inhibiting effect and the amount of lymphoid tissue declines because of the immunosuppression (Sjaastad, Hove & Sand, 2003). The stress induced cortisol secretion exists so that the body can handle and recuperate after an injury, physical activity or physiological strain (Wagner, 2010).

Cortisol in horses

Horses have a circadian pattern of their cortisol secretion with peak concentrations between 06-09 am and the lowest concentrations between 06-09 pm (Alexander & Irvine, 1998a). The concentration of the peak period is approximately doubled from that of the trough (Irvine & Alexander, 1994). Horses also have an episodic release of cortisol during the day with

approximately one peak per hour and the fluctuations are greatest during the peak period and more flattened during the trough (Toutain *et al*, 1988).

But the circadian rhythm can easily be disturbed by many factors like a different handling or environment than the one the horse is used to (Irvine & Alexander, 1994). When changing the environment, and thus putting the horse through stress, the cortisol level during the circadian trough rises whereas the peak concentration stay unaffected and the circadian pattern is hence gone (Irvine & Alexander, 1994). In short term the functions of a cortisol rise are beneficial for the horse, to cope with the environment, and if the stressor is not persistent a normal horse recovers and gets back to a normal circadian rhythm quickly (Alexander & Irvine, 1998a).

The plasma cortisol level normally changes with the age of the horse. During the first year of life great changes occur connected to the stress of weaning and growing (Fazio *et al*, 2009). Older horses are also more prone to diseases like Cushing's syndrome which alters both the episodic and circadian rhythm in horses (Orth *et al*, 1982). Equine Cushing's disease is most often originated in an adenoma of the *pars intermedia* of the pituitary gland but can also originate from the anterior lobe in rare cases (van der Kolk, 1997). The adenoma stimulates the production of ACTH and more cortisol from the adrenal glands is thus secreted (van der Kolk, 1997). The circadian rhythm is lost and the cortisol is constantly high in a horse with Cushing's disease (van der Kolk, 1997).

If a horse is put under chronic stress the normal circadian and episodic variation disappears and even small changes of the cortisol levels, but over long periods of time, can have great impact on the body. The horses get a decreased immune system and can become sick very easily. Studies have shown that horses under chronic stress caused by social instability lose weight and are more prone to get upper respiratory tract infections (Alexander & Irvine, 1998b).

Racehorses in training, which have a very structured and artificial environment surrounding them, can also show a circadian cortisol rhythm. As long as the horse is accustomed and relaxed in its environment the circadian rhythm can occur (Irvine & Alexander, 1994). A race, or just the excitement before a race, of course increases the plasma cortisol concentrations but the horses quickly recover and the day after the race the cortisol concentrations are back to normal (Alexander & Irvine, 1998a). Horses who are less trained can have higher plasma cortisol concentrations just after a race than more fit racehorses (Mircean *et al*, 2007) but the morning after compensate with lower cortisol levels than normal (Alexander & Irvine, 1998a). This is likely due to the effects of negative feedback on the pituitary and hypothalamus (Alexander & Irvine, 1998a).

Cortisol response to opioids

Studies have shown that plasma cortisol is affected by administration of synthetic opioids, like morphine and methadone, but how the cortisol release is affected varies widely (Pechnick, 1993). How the plasma cortisol is affected depends on what opioid is used, the species and the treatment protocol tested. In for example goats the plasma cortisol concentration rises after i.v. and i.m. administered buprenorphine but the response is not the

same three weeks later (Ingvast-Larsson *et al*, 2007). The plasma cortisol concentration have also shown to rise after administration of opioids to cats (Borrell, Llorens & Borrell, 1975), rats (Guaza *et al*, 1979) and dogs (Ingvast-Larssom *et al*, 2010). In humans the cortisol response generally decreases after opioid treatment (Vuong *et al*, 2010) and similar results have been seen in cattle with a lowered plasma cortisol concentration after opioid administration (Nanda, Dobson & Ward, 1992). Studies on this have not been performed in horses. How horses respond with their plasma cortisol concentration after administration of opioids is unclear and remains to be investigated.

Pain Assessment

Indicators for stress and pain in horses

To assess and measure pain in animals objectively is difficult because it is only possible to measure the indirect effects of pain in the body. The pain itself is in fact a subjective sensation and the extent of it is only known by the individual itself. But there are different ways to try to assess pain in animals and they can be divided into three groups; observation of behaviour, measurement of physiological parameters indicating sympathetic activation and measurement of plasma concentrations of factors indicating sympathetic activation (Radostits *et al*, 2007)

Examples of substances released into the blood during sympathetic activation are epinephrine, norepinephrine, non-esterified fatty acids, ACTH and plasma cortisol. Another substance that can be measured is serotonin. Epinephrine and norepinephrine are difficult and expensive to measure (Radostits *et al*, 2007) but the concentrations are significantly increased in the blood during acute pain and acute conditions (Ayala *et al*, 2011). Increased concentration of plasma non-esterified fatty acids is not very specific to pain, so that is not the best factor to be measured (Radostits *et al*, 2007). Serotonin can be measured in lower concentrations than normal during acute conditions and pain but is slightly increased in more chronic conditions (Ayala *et al*, 2011). The release of cortisol is very strongly connected to stressful and painful stimuli in horses (Ayala *et al*, 2011). Cortisol concentrations can be measured in plasma, saliva, urine and faeces and is the factor most commonly used when trying to estimate stress and pain in animals. (Radostits *et al*, 2007). It is also often used to evaluate analgesia during different treatments and operative procedures in animals (Sibanda *et al*, 2006).

MATERIAL & METHODS

The Local Ethical Committee in Uppsala, Sweden, approved the care of the animals and the experimental design.

Animals and Experimental Procedure

In this study eight clinically healthy warm-blood trotters, six mares and two geldings, belonging to the Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden, were used. They weighed between 444 to 586 kg and were all adults between 5 to 20 years of age.

The horses were housed in individual boxes and were fed with hay; they got fed after the 3-hour blood sample by the stable personnel, and had continuous access to water from water

drinkers. During the study period the horses were indoors but before and after the study they also went outdoors.

One hour before insertion of the venous catheters topical analgesia (Emla®, Astra Zeneca, Södertälje, Sweden, 25 mg/g lidocaine and 25 mg/g prilocaine) was applied in the area. Two catheters (14Ga, 12 cm Milacath®, Mila International Inc, Erlanger, USA) were inserted in each horse, one in each jugular vein. The catheters were sutured (Supramid 3-0, B. Braun Melsungen AG, Melsungen, Germany) with two or three isolated sutures and one catheter in each horse was connected to an extension tube (Discofix® C-3, B. Braun, Melsungen, Germany). The catheter with the extension tube was used for blood sampling and the one without was used for the drug administration and was then removed. The catheters were inserted approximately 1 h before the drug administration.

Drugs, Study Design and Blood Sampling

The study design used was a randomized, placebo controlled and blinded study with a *cross-over* design. There was at least a one week *wash out*-period between the treatment protocols.

All horses were treated with four different protocols; methadone (1), methadone + acepromazine (2), methadone + detomidine (3) and physiological saline solution (4). The treatment protocols looked like this;

1.) Methadone hydrochloride (Metadon recip, Recip, Solna, Sweden) administered i.v. during 5 minutes at a dose of 0.2 mg/kg together with physiological saline solution (Natriumklorid, Fresenius Kabi, Uppsala, Sverige, 9 mg/mL) administered i.m. in the neck right before the i.v. administration.
2.) Methadone hydrochloride administered i.v. at a dose of 0.1 mg/kg together with acepromazine (Plegicil® vet., Pharmaxin, Helsingborg, Sweden) administered i.m. in the neck at a dose of 0.05 mg/kg.
3.) Methadone hydrochloride administered i.v. at a dose of 0.1 mg/kg together with detomidine (Domosedan® vet., Orion Pharma Animal Health, Sollentuna, Sweden) administered i.m. in the neck at a dose of 0.01 mg/kg.
4.) Physiological saline solution administered i.v. and i.m. in the neck. All the i.v. administrations consisted of 20 mL, whether or not drugs or just saline solution was given.

Blood samples were taken from the jugular vein at the time of catheterization (this is the 0-sample) and thereafter 5, 11, 20, 30, 45, 60 and 90 minutes and 2, 3, 6, 10 and 22 hours after the drug administrations in all study groups. The 0-sample was taken at about 08-08.30 am and the first sample after the drug administrations at about 9 am and the last samples were taken at approximately 07 am the day after. The catheter and extension tube was flushed with 8-10 mL of physiological saline solution after every sampling. During every blood sampling 10 mL of blood was collected into tubes containing heparin which were immediately put on ice. The blood was then centrifuged for 10 minutes at 1500 ***g*** at 0 °C. The plasma, 2x3 mL, was then transferred to pre-chilled plastic tubes and stored at -80 °C until hormone analyses.

Cortisol Assay

Plasma cortisol was measured using a commercial cortisol enzyme-linked immunosorbent assay (ELISA) kit (DEMEDITEC, Kiel, Germany) developed for humans. The assay was based on competitive binding of antibodies. The ELISA kit consisted of a number of plates with 96 microtiter wells on each plate. All of the microtiter wells were coated with antibodies directed towards an antigenic site on the cortisol molecule. The cortisol of the horse plasma, standard or control samples then had to compete with an enzyme-linked conjugate (Cortisol-horseradish peroxidase conjugate) for binding to the coated antibodies. After incubation the conjugate was washed off and the remaining bound conjugate were inversely proportional to the cortisol concentration in the horse plasma. Substrate solution was added and a colour developed because of the substrate binding to the enzyme on the conjugate. Stop solution was finally added that inhibits further colouring. The colour intensity developed was inversely proportional to the cortisol concentration of the patient, standard and control samples.

The detection range of the assay was between 20-800 ng/mL. The cross reactivity of the antibodies in the assay was as follows; cortisol 100%, corticosterone 45%, progesterone <9%, deoxycortisol <2%, dexamethazone <2%, estriol <0.01%, estrone <0.01% and testosterone <0.01%. The intra-assay coefficient of variation of the assay when used in humans, according to Demeditec, is shown in *Table 1* and the inter-assay coefficient of variation is shown in *Table 2*.

Table 1. The Intra Assay Variation of Demeditec cortisol ELISA kit for human plasma, n=20 for every sample

Sample	Mean cortisol conc. (ng/mL)	%CV
1	43.5	8.1
2	226.5	3.2
3	403.6	5.6

Table 2. The Inter Assay Variation of Demeditec cortisol ELISA kit for human plasma, n=20 for every sample

Sample	Mean cortisol conc. (ng/mL)	%CV
1	55	6.6
2	209	7.7
3	361	6.5

Assay procedure

The plasma was first thawed on ice and the cortisol ELISA kit was getting time to reach room temperature. Standard, control and plasma samples (20 µL), where each sample was paired, were put into their coated wells. Enzyme conjugate (200 µL) was then put into each well and the wells were thoroughly mixed during 60 minutes of incubation at room temperature. The content was shaken out and the wells were washed with wash solution (300 µL) five times. Substrate solution (100 µL) was then put into each well followed by 15 minutes of incubation and addition of stop solution (100 µL) to each well. The absorbance was then read by a

microtiter plate reader (Wallac Victor², 1420 Multilabel Counter, Wallac Sverige AB) at 450 nm within 10 minutes.

Statistical Analysis

For all the absorbance values of standard, control and plasma samples mean, standard deviation (SD) and % coefficient of variation (%CV) were calculated (CV=mean/SD). Mean, SD and %CV were also calculated for the cortisol concentrations obtained in the validation and for all the standards. A logarithmic standard curve was made for each ELISA plate (Microsoft Excel version 14.0.6129.5000, Microsoft Corporation). To calculate the concentration of cortisol in each sample a 4-Parameter Logistics curve fit was used (ReaderFit, Hitachi Solutions America, Ltd. 2012). All sample concentrations were statistically analysed using Minitab® Statistical Software (Minitab®, version 16.1.0, 2010 Minitab Inc., USA) and subjected to a Kruskal-Wallis Test since the data did not follow a normal distribution. The significance level was set at $p < 0.05$.

RESULTS

Validation of the Cortisol ELISA Kit in Horses

The cortisol ELISA kit used was developed for humans and thus not tested nor validated for horses. Therefore, the first test runs were done to validate this method in horses and to see that the samples were within the detection range of the cortisol ELISA kit. Nine different samples, with already determined cortisol concentrations using another method, liquid chromatography-mass spectrometry (LC-MS/MS), were used for this purpose and tested with the cortisol ELISA kit. The results are shown in *Table 3*.

Table 3. Validation of the cortisol ELISA kit. Plasma samples with already determined cortisol concentration using another method, LC-MS/MS, were tested with the cortisol ELISA kit. %CV was calculated to show the difference between the methods

ELISA conc. (ng/mL)	LC-MS/MS conc. (ng/mL)	%CV
92.3	92	0.2
71.1	78	6.5
64.2	52	14.8
43.8	36	13.9
43.5	38	9.6
43.3	44	1.1
39.7	46	10.4
23.4	26	7.6

Spiked samples were also used to measure the accuracy of the detection range of samples with concentrations more than 90 ng/mL in the cortisol ELISA kit. A sample with very low cortisol concentration (3.5 ng/mL) from this study was used and spiked, with solutions containing a known concentration of cortisol, in a ratio of 1:1. The results are shown in *Table 4*.

Table 4. Cortisol concentration of spiked samples tested with the cortisol ELISA kit. A plasma sample with very low (3.5 ng/mL) cortisol concentration was mixed together with samples with known cortisol concentrations in a ratio of 1:1. %CV was calculated to show the difference between the obtained and the spiked cortisol concentrations

Obtained ELISA conc. (ng/mL)	Spiked conc. (ng/mL)	%CV
91.6	100	6.2
190.1	200	3.6
400.6	400	0.1

For each ELISA plate paired standard samples were included and a standard curve was made from them. To make a quality control of the method, and the person performing it, all the %CV from the standards were compared and the result can be seen in *Table 5*.

Table 5. Range of the %CV for all the paired standard samples tested on all the ELISA plates, n=14 for every concentration

Standard conc. (ng/mL)	Range %CV
0	0.4 – 3.4
20	0.2 – 6.9
50	0.01 – 6.1
100	0.8 – 7.0
200	0.03 – 11.0
400	0.2 – 9.8
800	0.2 -5.7

The inter-assay coefficient of variation was also calculated from all the concentrations obtained from the standards of all the test runs. This result is shown in *Table 6*.

Table 6. The inter-assay coefficient of variation from all the standard samples tested, n=14 for every concentration

Standard conc. (ng/mL)	Mean ELISA conc. (ng/mL)	%CV
0	0	
20	20	2.5
50	49	3.4
100	102	3.1
200	197	3.1
400	409	2.7
800	787	3.6

Plasma Cortisol Concentrations

Before treatment, whether or not drugs or just control were given, the plasma cortisol concentration was 67.6 ± 1,06 ng/mL (mean ± SD). As seen in *Figure 1*and *Table 7* the changes in plasma cortisol concentration only occur the first few hours after administration,

thereafter no difference is seen between the treatment protocols. After administration of methadone there was a statistical significant rise in plasma cortisol concentration compared to the control value consisting of administration of physiological saline solution ($p < 0.05$) (*Figure 2*). There was also a significant rise in the plasma cortisol concentration after administration of methadone + detomidine ($p < 0.05$) (*Figure 3*) but no significance was seen after administration of methadone + acepromazine (*Figure 4*).

Table 7. Mean (±SD) cortisol concentration for all the horses (n=8) for the four different treatments with methadone (0.2 mg/kg i.v.) + saline (i.m.), saline (i.v. and i.m.), methadone (0.1 mg/kg i.v.) + acepromazine (0.05 mg/kg i.m.) and methadone (0.1 mg/kg i.v.) + detomidine (0.01 mg/kg i.m.)

Time (h)	Cortisol conc. (ng/mL) Saline	Cortisol conc. (ng/mL) Methadone + Saline	Cortisol conc. (ng/mL) Methadone + Acepromazine	Cortisol conc. (ng/mL) Methadone + Detomidine
0	67 ± 18	67 ± 21	69 ± 20	67 ± 21
0.08	48 ± 13	52 ± 23	48 ± 16	63 ± 41
0.2	48 ± 14	54 ± 16	45 ± 18	63 ± 34
0.33	46 ± 12	69 ± 13	44 ± 18	60 ± 28
0.5	45 ± 10	83 ± 16	44 ± 19	61 ± 26
0.75	49 ± 13	94 ± 27	52 ± 19	56 ± 24
1	46 ± 14	93 ± 26	61 ± 29	53 ± 24
1.5	42 ± 17	70 ± 16	53 ± 21	49 ± 22
2	43 ± 15	59 ± 19	45 ± 17	50 ± 31
3	43 ± 11	47 ± 19	40 ± 11	52 ± 20
6	32 ± 18	32 ± 13	29 ± 9	43 ± 29
10	20 ± 14	23 ± 15	21 ± 13	24 ± 16
22	58 ± 15	71 ± 26	66 ± 26	70 ± 18

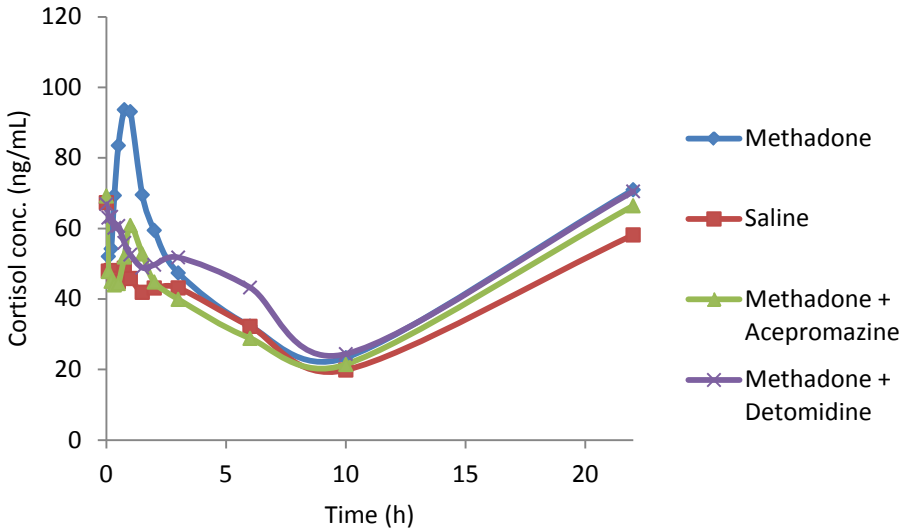

Figure 1. Mean plasma cortisol concentration for all the horses (n=8) for the four different treatments with methadone (0.2 mg/kg i.v.) + saline (i.m.), saline (i.v. and i.m.), methadone (0.1 mg/kg i.v.) + acepromazine (0.05 mg/kg i.m.) and methadone (0.1 mg/kg i.v.) + detomidine (0.01 mg/kg i.m.

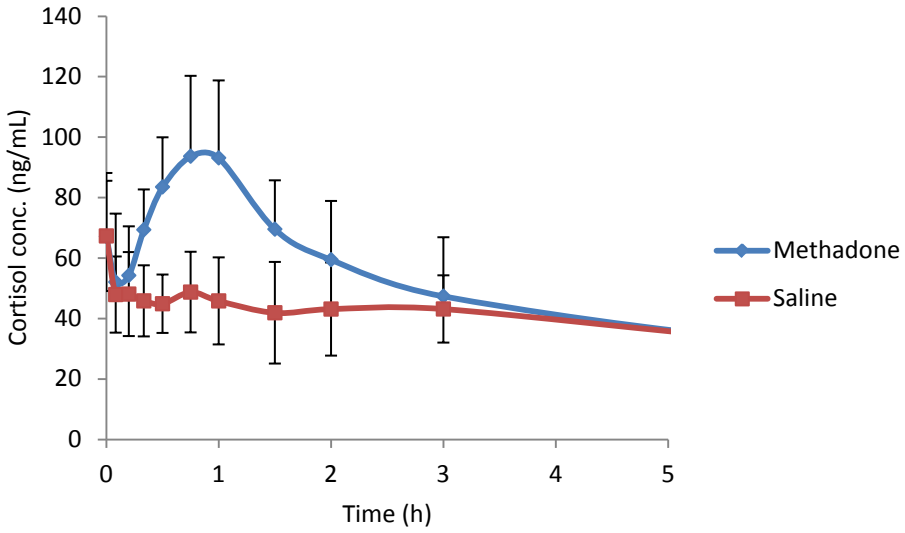

Figure 2. Mean (±SD) plasma cortisol concentration the first 5 hours for all the horses (n=8) after administration of methadone (0.2 mg/kg i.v.) + saline (i.m.) and saline (i.v. and i.m.). A statistical significant difference was seen ($p<0.05$).

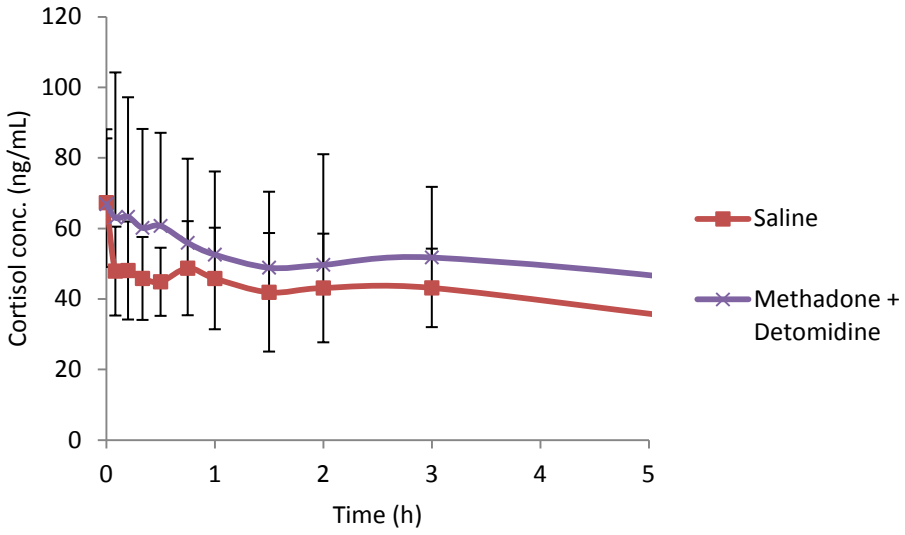

Figure 3. Mean (±SD) plasma cortisol concentration the first 5 hours for all the horses (n=8) after administration of methadone (0.1 mg/kg i.v.) + detomidine (0.01 mg/kg i.m.) and saline (i.v. and i.m.). A statistical significant difference was seen (p<0.05).

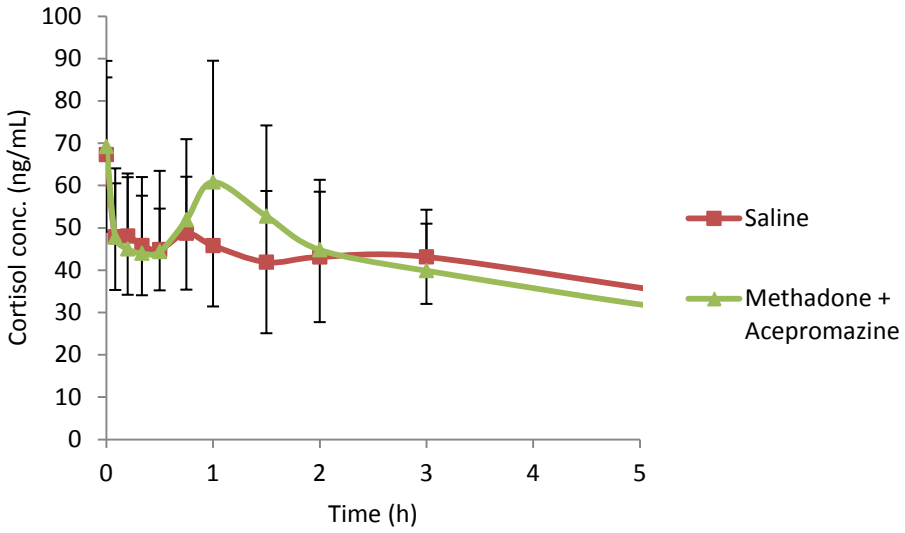

Figure 4. Mean (±SD) plasma cortisol concentration the first 5 hours for all the horses (n=8) after administration of methadone (0.1 mg/kg i.v.) + acepromazine (0.05 mg/kg i.m.) and saline (i.v. and i.m.). No statistical significance was seen (p > 0.05).

The individual difference between the horses in how they responded with their cortisol release to the different treatment protocols was very noticeable. This is shown in *Figure 5* where all the horses cortisol concentrations are shown after methadone administration. Even though this great individual difference existed there was still a statistical significance between saline, methadone and methadone + detomidine treatments.

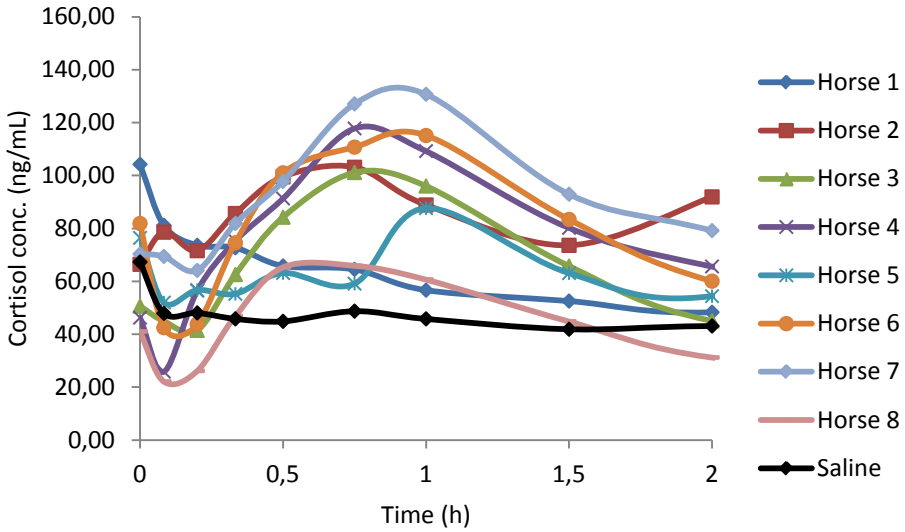

Figure 5. The individual cortisol response in the eight horses (n=8) the first 2 hours after administration of methadone (0.2 mg/kg i.v.) + saline (i.m.) compared to the mean cortisol concentration after administration of only saline (i.v. and i.m.). It is noteworthy to mention that horse 5 only got half the dose (0.1 mg/kg i.v.) of methadone than the rest of the horses.

Unfortunately horse 5 got sick with esophageal obstruction during the study so the last two blood samples during methadone + detomidine treatment was left out and it also only got half the dosage of methadone (i.e. 0.1 mg/kg i.v.) than the rest of the horses during methadone + saline treatment.

DISCUSSION

Before evaluating the results it is important to consider the cross-reactivity with corticosterone, which was up to 45% with the cortisol ELISA kit. Cortisol is the major glucocorticoid in horses but they also have corticosterone, the ratio between cortisol, cortisone and corticosterone have shown to be 16:8:0.5 (Zolovick, Upson & Eleftheriou, 1966). It is important to have the cross-reactivity in mind when evaluating the cortisol results even though the major part consists of cortisol and not corticosterone.

The Demeditec cortisol ELISA kit was developed for humans but not tested nor validated for horses. Hence, a validation for the use in horses was done. Plasma samples from horses with already known cortisol concentrations, determined by another method (LC-MS/MS), were analysed with the cortisol ELISA kit and %CV was calculated and the method analysed. The %CV obtained were between 0.2-14.8 % for the samples with cortisol concentrations ranging between 26-92 ng/mL. This is generally counted as acceptable. Plasma samples with lower cortisol concentration than 20 ng/mL would need an extraction procedure or a different method. This is because the lowest standard in the kit was 20 ng/mL.

The spiked samples had a %CV between 0.1 - 6.2 %, which is considered acceptable, and the range analysed was between 100-400 ng/mL. All the plasma samples used in this study were within that range and the results can thus be considered reliable.

For each run with the cortisol ELISA kit mean, standard deviation and %CV were calculated. This was done for both plasma samples and standard samples. The %CV was compared for all the paired standards and the range for all the concentrations were between 0.01 – 11.0 % and this is a good result indicating that the method and the procedure were very precise within every run. All the concentrations obtained from the standards were also compared so that an in between test mean, standard deviation and %CV could be calculated. This result was between 2.5-3.6 % indicating that the variability between the test runs was quite low as well.

This study showed that after i.v. administration of methadone plasma cortisol concentration increased in horses. This change mainly occurred between 0.5-2 h after the injections, thereafter the concentrations declined to levels as before the study started. After 2 h all the plasma cortisol concentrations more or less followed the same pattern in all horses indicating that the effects of the different drugs had ceased or decreased drastically. Methadone in horses, after i.v. administration, has shown to have a quite short elimination half-life. In one study the elimination half-life was approximately 1 h (Linardi *et al*, 2012). With such a short elimination half-life the rapid increase and thereafter decrease of plasma cortisol is probably connected to the short stay of methadone in the blood. The plasma half-life of cortisol in horses can be up to 1,55 ± 0,33 h (Lassourd *et* al, 1996) which could make the effect of a cortisol release after methadone administration more prolonged. It is noteworthy to mention that the first sample, taken when the horses got their jugular catheters inserted, is always higher than the following sample taken 5 minutes after the drug administrations (and thus approximately 1 h after the catheterization). This could be due to the stress of getting the catheters inserted but also an effect of the circadian rhythm. The latter does not seem very likely because the first and second sample were taken between 8 and 9 am and the concentration should be rather similar at this time of day (Irvine & Alexander, 1994). However, the cortisol concentrations in samples taken the next day 22 hours after drug administration are comparable to the precatheterization samples which indicate that the circadian rhythm can be the cause after all.

When methadone was given alone it was at a dose of 0.2 mg/kg and when methadone was given in combination with either acepromazine or detomidine the dose was 0.1 mg/kg. The dosage of methadone used for horses varies but many recommend the dose 0.1 mg/kg i.v. (Clutton, 2010) but higher doses have also been suggested. Others say that 0.1 mg/kg methadone i.v. is the correct dose when given together with another drug, like acepromazine (Mason, 2004). Thus, the doses used in this study were not very high nor very low, they were the doses normally used in clinical situations.

The greatest statistical significant cortisol release in this study was when methadone was given alone. This is probably because of the higher dosage. Horse 5 only got half the dose of methadone (0.1 mg/kg instead of 0.2 mg/kg i.v.) and its cortisol response to methadone can be seen *in Figure 5*. Horse 5 had a somewhat different cortisol response, with lower cortisol concentrations, than the other horses but the cortisol response were still very individual so no major conclusions can be drawn from this. A statistical significant rise in plasma cortisol after methadone + detomidine administration was also seen, but the rise of the plasma cortisol concentration was not as pronounced as when methadone was given alone at a higher dose.

However, no statistical significant rise in plasma cortisol was seen after methadone + acepromazine administration even though the same dose of methadone was used as in the methadone + detomidine administration. A theory why the plasma cortisol concentrations increased when methadone was given together with detomidine, but not together with acepromazine, may be that acepromazine itself affects the plasma cortisol levels in some way. Acepromazine is, as mentioned before, an α_1-adrenoreceptor antagonist (Rang *et al*, 2007). It has been showed in humans that α_1-adrenoreceptor agonists increase the levels of ACTH and cortisol in the blood and that α_1-adrenoreceptor antagonists decrease the ACTH levels in the blood (al-Damluji & Francis, 1993). This may be the reason why the cortisol levels did not rise after administration of methadone + acepromazine in these horses.

In humans opioids generally decrease the plasma cortisol levels. This occurs both after a single injection and after more long-term abuse of opioids (Vuong *et al*, 2010). Tolerance develops quickly, within 12-24 h, and higher and higher doses are needed to provide good analgesia (Rang *et al*, 2007). There has been seen in heroin addicts that the normal circadian cortisol rhythm is lost and that they have a constant level of cortisol in the blood throughout the day instead (Vuong *et al*, 2010). This can lead to adrenal insufficiency or a decreased glucocorticoid response to acute stress. Studies have shown that opioids can disrupt the cortisol release via the hypothalamus, the pituitary and the adrenal glands (Vuong *et al*, 2010).

Experiments in rhesus monkeys have shown that, when given an opioid antagonist, a stimulation of the HPA axis can occur. The stimulation that occurred was that more ACTH and thus cortisol were released (Williams *et al*, 2003). Other experiments in rhesus monkeys have shown an increase in ACTH and cortisol after a dose of an opioid agonist. In these experiments only the agonists for the κ-receptor stimulated the HPA axis (Pascoe *et al*, 2008).

In cats, a single injection of morphine increases the plasma cortisol levels (Borrell, Llorens & Borrell, 1975). However, after 14 days of daily intraperitoneally (i.p.) injections of morphine the cortisol levels in the adrenal glands have decreased significantly and after 30 days of daily injections the plasma cortisol level decreases as well (Guaza *et al*, 1979).

Studies have also been done in rats that have shown that a single injection i.p. of morphine induces a rise in the plasma corticosterone levels. Just as in cats, after daily injections of morphine the plasma corticosterone levels decrease. Unlike cats the levels are significantly lower after just 6 days of injections and the corticosterone levels in the adrenal glands are also lower than before the treatment (Guaza *et al*, 1979).

Similar results can be seen in dogs, a single injection of the opioid agonist-antagonist butorphanol elevates the plasma cortisol concentrations compared to dogs only given saline (Fox *et al*, 1998). The plasma cortisol concentration also increases when dogs are given methadone via i.v. administration (Ingvast-Larsson *et al*, 2010). But the increase of cortisol is only temporary and within approximately 3 hours the levels are back to normal again. A subcutaneous administration of methadone has shown not to increase the plasma cortisol levels, but this could be due to the fact that a subcutaneous injection does not produce very

high concentrations of methadone in the blood (Ingvast-Larsson *et al*, 2010). Ingvast-Larsson *et al* suggest that cortisol is not a good parameter when assessing analgesic efficacy of methadone in dogs because of the release of cortisol by the drug itself.

The response to morphine in cows is different to that in the other species discussed above because a single dose of morphine i.v. decreases the plasma cortisol levels in cows. When given naloxone, an opioid antagonist, the cortisol levels increase. This could be a sign of suppressive control of opioids on the HPA axis in dairy cows. In the study by Nanda, Dobson & Ward a cow with chronic lameness, and thus under chronic stress, responded in the opposite way than the rest of the cows. Why this happened is unclear but it could be because different receptors are involved during chronic stress or a modulation of the normal HPA axis (Nanda, Dobson & Ward, 1992).

Before evaluating the analgesia of a specific drug during a procedure it is important to have in mind how that specific drug affects the cortisol release in the species tested. When having a well thought through experiment the plasma cortisol can be useful as an indicator of stress or pain as potential biases from observing behaviour are avoided (Hart, 2012) and the release of cortisol is very strongly connected to stressful and painful stimulus in horses (Ayala *et al*, 2011).

Conclusions

The validation of the cortisol ELISA kit showed that it works well on horse plasma in the concentration range between 20-400 ng/mL. Another conclusion in this study is that the within and between assay variability were very low which indicate that the concentrations obtained in this study were rather accurate.

The results of this study show that i.v. administered methadone itself, either alone or together with detomidine, induces the release of plasma cortisol in horses. However, no increased cortisol concentrations were seen after methadone + acepromazine treatment. To what extent methadone affects the plasma cortisol concentrations in horses thus seems to be dependent on with what substance methadone is used together with. These results indicate that the plasma cortisol concentration may not be a useful parameter for stress or pain when evaluating analgesia of methadone in horses.

ACKNOWLEDGEMENTS

Thank you Lena Olsén, Åsa Ohlsson Andersson and Melker for all the help and for hovering over me in the laboratory pushing me to achieve such good results! Without you I would not have obtained my r-squares of 0.9999 on my 4 parameter logistics curve!

Furthermore I would like to thank Stiftelsen Hästforskning for funding the project.

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