Dexamethasone in Horses

Safe Usage from an Anti-Doping and Medication Perspective

Carl Ekstrand
Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health
Uppsala

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Abstract

Equine athletes and companion horses are commonly treated with glucocorticoids such as dexamethasone. Care of sick or injured animals is vital for the animal welfare. To protect the integrity of equine sport, legal therapeutic substances are regulated during competition. The overall aim of this thesis was to provide quantitative information of dexamethasone exposure and response in Standardbreds and to relate this information to medication and anti-doping methodology.

Dexamethasone-21-isonicotinate suspension administered intramuscularly and dexamethasone sodium phosphate solution administered both intravenously and intra-articularly were used in experiments. Dexamethasone was quantified in plasma, urine and synovial fluid by means of UHPLC-MS/MS. Cortisol response, IL-1β response, joint circumference response, local skin temperature response and lameness response were used as biomarkers. Dexamethasone plasma- and synovial fluid concentration-time courses were characterised by means of compartment modelling. The response-time courses were described by fitting a turnover model with an inhibitory function to experimental biomarker response data.

Dexamethasone exposure was described in plasma and synovial fluid. In the Standardbreds studied, median dexamethasone plasma clearance was 0.51 $L \cdot h^{-1} \cdot kg^{-1}$ and median volume of distribution (at steady state) was 1.58 $L \cdot kg^{-1}$. The median half-life in plasma and synovial fluid was 2.4 h and 1.3 h, respectively. After intramuscular administration of dexamethasone-21-isonicotinate, mean terminal half-life was 39 h due to slow release into plasma from the injection site. The dexamethasone concentration was 4- to 15-fold higher in urine than in plasma. A circadian cortisol baseline was described using a cosine function and cortisol response was characterised. Median potency value and for cortisol response in plasma was 0.039 $ng \cdot mL^{-1}$ and the efficacy value was 0.92. Dexamethasone inhibited lameness and synovial fluid IL-1β release at doses lower than the approved. The median calculated synovial fluid concentration that suppressed lameness response by 50 % was 17 $ng \cdot mL^{-1}$. The tentative potency value for IL-1β response was 19 $ng \cdot mL^{-1}$.

The information provided in this thesis can be used to improve future medication protocols and anti-doping controls which could improve animal welfare.

Keywords: pharmacokinetics, pharmacodynamics, potency, efficacy, turnover model, integrative pharmacology, quantitative pharmacology, corticosteroids, glucocorticoids

Author's address: Carl Ekstrand, SLU, Department of Biomedical Sciences and Veterinary Public Health, P.O. Box 7028, 750 07 Uppsala, Sweden
E-mail: Carl.Ekstrand@slu.se

Dedication

To Karin, Alice and Edvin.

Future generations will judge us not by what we say, but what we do.
Ellen Johnson Sirleaf

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ekstrand, C., Bondesson, U., Gabrielsson, J., Hedeland, M., Kallings, P., Olsén, L. & Ingvast-Larsson, C. (2015). Plasma concentration-dependent suppression of endogenous hydrocortisone in the horse after intramuscular administration of dexamethasone-21-isonicotinate. *Journal of Veterinary Pharmacology and Therapeutics.* 38(3), 235-242.

II Ekstrand, C., Ingvast-Larsson, C., Olsén, L., Hedeland, M., Bondesson, U. & Gabrielsson, J. (2016). A quantitative approach to analysing cortisol response in the horse. *Journal of Veterinary Pharmacology and Therapeutics.* 39(3), 255-263.

III Ekstrand, C., Bondesson, U., Giving, E., Hedeland, M., Ingvast-Larsson, C., Jacobsen, S., Löfgren, M., Moen, L., Saetra, T. & Ranheim, B. (2017). Disposition and effect of intraarticular administered dexamethasone on lipopolysaccharide-induced inflammation in the equine joint (*manuscript*).

IV Ekstrand, C., Ingvast-Larsson, C., Saetra, T., Giving, E., Moen, L., Rhodin, M. & Ranheim, B. (2017). Intraarticular dexamethasone in an equine model of inflammation: effect on lameness and local joint inflammation (*manuscript*).

The contribution of Carl Ekstrand to the papers included in this thesis was as follows:

I Shared responsibility for data analysis and interpretation. Wrote the manuscript and corresponded with the journal.

II Shared responsibility for planning and executing the experiment. Main responsibility of data analyses and interpretation of the results. Wrote the manuscript and corresponded with the journal.

III Main responsibility for planning and execution of the experiment, data analyses and interpretation of the results. Wrote the manuscript.

IV Main responsibility for planning and execution of the experiment data analyses and interpretation of the results. Wrote the manuscript.

Abbreviations

A, B	Pharmacokinetic macro parameters
A_{inhib}	Pharmacodynamic parameter
A_{LPS}	Amplitude of the LPS-challenge function
α, β	Slope factors (h^{-1})
α_{circ}	Amplitude of the circadian function
AAEP	American Association of Equine Practitioners
ARCI	Association of Racing Commissioners International
C_p	Plasma concentration of dexamethasone ($ng \cdot mL^{-1}$)
C_s	Synovial fluid concentration of dexamethasone ($ng \cdot mL^{-1}$)
C_t	Tissue concentration of dexamethasone ($ng \cdot mL^{-1}$)
CE	Clinical endpoint
Cl	Clearance ($L \cdot h^{-1} \cdot kg^{-1}$)
Cl_d	Inter-compartmental distribution parameter ($L \cdot h^{-1} \cdot kg^{-1}$)
DSP	Dexamethasone 21-phosphate disodium salt
DT	Detection time (*e.g.* h)
E_{max}	Maximum effect
EC_{50}	Concentration producing 50% of maximal effect (*e.g.* $ng \cdot mL^{-1}$)
EHSLC	European Horserace Scientific Liaison Committee
EPC	Effective plasma concentration (*e.g.* $ng \cdot mL^{-1}$)
EUC	Effective urine concentration (*e.g.* $ng \cdot mL^{-1}$)
F	Bioavailability
FEI	Fédération Equestre Internationale
I_{max}	Maximum inhibitory effect
ia	Intraarticular
$I(C_p)$, $I(C_s)$	Inhibitory drug mechanism functions
IC_{50}	Concentration producing 50 % of maximal inhibition (*e.g.* $ng \cdot mL^{-1}$)

IFHA	International Federation of Horseracing Authorities
IL-1β	Interleukin-1β
im	Intramuscular
Inf	The dose infused ($\mu g \cdot kg^{-1}$)
IPC	Irrelevant plasma concentration (*e.g.* $pg \cdot mL^{-1}$)
IUC	Irrelevant urine concentration (*e.g.* $pg \cdot mL^{-1}$)
iv	Intravenous
JC	Joint circumference (cm)
k	First order elimination rate constant (h^{-1})
k_a	First order absorption rate constant (h^{-1})
k_{in}	Zero order turnover rate for production of response (h^{-1})
k_{LPS}	Rate constant of the LPS-challenge function (h^{-1})
k_{mean}	Mean input rate (of cortisol)
k_{out}	Fractional turnover rate of response (h^{-1})
LL	Lameness locator
LPS	Lipopolysaccharide
MHH	Minimum head height
n	Sigmoidicity (slope) factor
PD	Pharmacodynamics
PK	Pharmacokinetics
PK/PD	Pharmacokinetic/pharmacodynamic
R	Response (*e.g.* biomarker value)
R_{ss}	Dexamethasone concentration-response relationship
RSD	Relative standard deviation
RT	Rectal temperature (º C)
S_{LPS}	LPS-challenge function
SF	Safety (uncertainty) factor
SL	Screening limit (*e.g.* $ng \cdot mL^{-1}$)
ST	Local skin temperature (over the challenged joint) (º C)
t	Time (h)
t_0	Peak time of the circadian function (h)
$t_{1/2}$	Effective half-life (h)
$t_{1/2\alpha}$	Half-life of initial slope (h)
$t_{1/2\beta}$, $t_{1/2z}$	Terminal half-lifes (h)
UHPLC-MS/MS	Ultra high performance liquid chromatography-tandem mass spectrometry
V_{ss}	Volume of distribution (at steady state) ($L \cdot kg^{-1}$)
V_c	Volume of the central compartment ($L \cdot kg^{-1}$)
V_t	Tissue volume ($L \cdot kg^{-1}$)
WT	Withdrawal time (*e.g.* h)

1 Introduction

1.1 Glucocorticoids in a historic perspective

In 1950, Edward Calvin Kendall, Tadeus Reichstein and Philip Showalter Hench were awarded the Nobel Prize for Physiology and Medicine "for their discoveries relating to the hormones of the adrenal cortex, their structure and biological effects" (Nobel Media, 2014). The main group of hormones originating from the adrenal cortex are the corticosteroids. They can be further divided into the subgroups mineralocorticoids and glucocorticoids. Due to their effective and potent anti-inflammatory and immune-suppressive properties, glucocorticoids rapidly became essential in treatment of rheumatoid arthritis in humans (Kendall, 1951a, b, 1959).

Glucocorticoids such as dexamethasone are now frequently used in both human and veterinary medicine. Several pharmaceutical preparations and routes of administration are registered for use in horses (Medical Products Agency, 2010, 2015). In the 1960s and 1970s, the clinical response to glucocorticoids administered intraarticularly (ia) to horses with joint disease, *e.g.* osteoarthritis was investigated (Trussel, 1965; Van Pelt & Riley, 1967; Houdeshell, 1969, 1970; Van Pelt *et al.*, 1970; Van Pelt *et al.*, 1971; Vernimb *et al.*, 1977). In these early studies, different substances and pharmaceutical preparations were used. Based on the results, it was concluded that horses responded satisfactorily to the treatment. The onset of response was reported within two days, the clinical response was positive in up to more than 90 % of the treated horses and the outcome of treatment was in general terms referred to as *e.g.* "good" or "excellent". More recent studies have also reported the response to glucocorticoid treatment in horses to be 27-88 % successful (Labens *et al.*, 2007; Kay *et al.*, 2008; Brommer *et al.*, 2012; de Grauw *et al.*, 2016). Those studies include both prospective and retrospective studies on joint disease as well as experimental models of joint inflammation. The response to

glucocorticoids of inflammatory airway disease, *e.g.* recurrent airway obstruction, has also been extensively studied. In the literature, there is strong evidence of a reduction in both clinical signs and biomarker response, indicating improved airway function, after both topical and systemic administration of glucocorticoids (Ammann *et al.*, 1998; Robinson *et al.*, 2002; Picandet *et al.*, 2003; Robinson *et al.*, 2003; Cornelisse *et al.*, 2004; Couetil *et al.*, 2006; DeLuca *et al.*, 2008; Leclere *et al.*, 2010). A response to glucocorticoid treatment of other inflammatory conditions in horses has also been described (Frauenfelder *et al.*, 1982; McCue *et al.*, 2003; Christoffersen *et al.*, 2012).

1.2 Pharmacokinetics and pharmacodynamics

1.2.1 General concepts

One description of pharmacology is that it is the study of how drugs interact with the body. Two disciplines within pharmacology are pharmacokinetics (PK) and pharmacodynamics (PD). One prerequisite for any drug response is that the drug must be available at the site where it will exert its action. If the drug is designed for topical administration, it may be distributed locally to this (local) site. If the site of action is out of reach the drug must be transported to the target tissue by the blood stream, regardless what route of administration that was used. If the drug not is injected into the blood it must be absorbed before it is distributed by the circulation to the site where it exerts its action. The drug most commonly also must be transported to the site where it is removed (eliminated) from the body or metabolised. Hence, PK is the study of absorption, distribution, metabolism and elimination of a drug. In other words, PK can be described as what the body does to the drug (Benet & Zia-Amirhosseini, 1995).

One description that has been used for PD is what the drug does to the body (Holford & Sheiner, 1982; Derendorf & Meibohm, 1999). Most drugs act by interaction with a biological target to produce a response. This target might be an enzyme, a receptor located on the cell surface or within the cell, a carrier (transport) molecule, an ion channel *etc*. The resulting PD can be reported in qualitative and quantitative terms. Qualitative terms describe how a biomarker or clinical response changes using a categorical outcome (*e.g.* treatment was poor, fair, good, *etc*.) or relative to a baseline (*e.g.* biomarker value was 60 % lower after treatment). Quantitative terms for PD describe the change in biomarker response numerically, *i.e.* PD (model) parameter estimates are reported. Examples of PD-parameters are potency values (EC_{50}/IC_{50}-value), which is the concentration that generates 50 % of maximum response, efficacy

values (E_{max} and I_{max}) and information about the concentration-response relationship (sigmoidicity factor, *n*). The ideal potency value would be the free concentration at the target site. This concentration is often not measured for practical reasons. Rather, the blood (*e.g.* plasma or serum) or synovial fluid drug concentration is often used as a surrogate concentration.

Three relationships should be considered to include in PK/PD analysis: Drug concentration *versus* time, drug response *versus* time and drug response *versus* drug concentration. The use of PK/PD analyses and integration of these relationships simplifies interpretation of a data set when the concentration-time course is different from the response-time course *e.g.* due to a delay because of physiological processes. For example if the response to a drug is dependent on altering gene expression in order to influence the release of a protein. Pharmacodynamic modelling by means of *e.g.* effect compartment models and turnover models relate drug concentration to the response and are examples of integration of the concentration-time and response-time courses (Segre, 1968; Sheiner *et al.*, 1979; Dayneka *et al.*, 1993; Levy, 1994). The use of quantitative methods also makes it possible to build models to simulate concentration-response relationships or time courses beyond what has been studied in the experiment. This reduces the numbers of animals and is favourable from a scientific and economic perspective. Attempts to quantify the response to glucocorticoid exposure in humans and rodents by means of biomarkers have successfully been done by Jusko and co-workers, affiliated to University at Buffalo, the State University of New York (Dayneka et al., 1993; Lew & Jusko, 1993; Jusko, 1995; Chakraborty *et al.*, 1999; Krzyzanski *et al.*, 2000; Chakraborty *et al.*, 2005; Jin & Jusko, 2009)

1.2.2 Pharmacokinetics of dexamethasone in the horse

The plasma dexamethasone exposure has been described and some studies also provide PK information on different pharmaceutical preparations in both Saddlebreds and Thoroughbreds (Toutain *et al.*, 1984; Cunningham *et al.*, 1996; Soma *et al.*, 2005; Authie *et al.*, 2010; Grady *et al.*, 2010; Soma *et al.*, 2013). Consequently, quantitative information and reported values of PK parameters *e.g.* bioavailability (*F*), clearance (*Cl*), volume (*V*) and rate constants for absorption and elimination are available. However, plasma dexamethasone exposure of after intramuscular (im) administration of dexamethasone-21-isonicotinate slow release suspension has not been published. In addition, there is sparse information on plasma dexamethasone disposition in Standardbreds.

For synovial fluid, there is no information available on the exposure of dexamethasone following ia administration.

1.2.3 Pharmacodynamics of dexamethasone in the horse

The response to dexamethasone has been described in qualitative terms using both clinical signs and other biomarkers of anti-inflammatory, metabolic, haematological and endocrine response (Osbaldiston & Johnson, 1972; Toutain et al., 1984; Lane *et al.*, 1990; Salles-Gomes *et al.*, 2003; Cornelisse et al., 2004; Soma et al., 2005; Cartmill *et al.*, 2006; Abraham *et al.*, 2009; Grady et al., 2010; Abraham *et al.*, 2011; Soma et al., 2013). However, none of those studies has presented convincing quantitative information based on PK/PD modelling. On the contrary, plasma glucocorticoid concentration has been suggested to be less important for the response than intracellular dexamethasone concentrations and therefore irrelevant (Lees *et al.*, 1990; Grady *et al.*, 2010). In contrast, in human medicine a clear plasma glucocorticoid concentration-response relationship has been described (Kong *et al.*, 1989; Lew & Jusko, 1993; Rohatagi *et al.*, 1996a; Rohatagi *et al.*, 1996b; Mager *et al.*, 2003). Moreover, it has been found that increasing the glucocorticoid doses during inhalation therapy also increases the cortisol response (*i.e.* decreasing the cortisol plasma concentration) in horses (Rush *et al.*, 1999).

Cortisol (endogenous hydrocortisone) response has frequently been used as a biomarker of response to dexamethasone exposure (Eiler *et al.*, 1979; Slone *et al.*, 1983; Toutain et al., 1984; MacHarg *et al.*, 1985; Soma et al., 2005; Abraham et al., 2009; Grady et al., 2010; Soma et al., 2013). However, the only quantitative information presented in those studies is a proposed dexamethasone threshold concentration in plasma (0.19 $ng \cdot mL^{-1}$) "switching off" cortisol production (Soma *et al*., 2005). In contrast, the glucocorticoid beclomethasone suppress cortisol response dose dependently (Rush *et al*., 1999). However, despite the similarity in changes over time of cortisol response and pleural pressure response in horses with airway obstruction treated with dexamethasone, there is no evidence that cortisol response is a suitable marker for the anti-inflammatory response to dexamethasone (Cornelisse *et al*., 2004; Soma *et al*., 2005).

1.3 Doping and medication control of equine athletes

1.3.1 Background, rules and legislations

Many substances may alter the performance of equine athletes and endanger animal welfare and fair competition and put the integrity of the sport at risk. Therefore, some substances (*e.g.* anabolic steroids) are banned from use in sport-horses by many regulatory organisations, *e.g.* members of *Fédération Equestre Internationale* (FEI) and the International Federation of Horseracing Authorities (IFHA) (FEI, 2017a; IFHA, 2017a). Finding of a banned substance

and/or its metabolite or prodrug in plasma or urine samples from a sport-horse is considered to be conclusive evidence for doping, so called zero tolerance policy. However, for animal welfare reasons, sick or injured animal must be given proper care, which includes medication with legitimate therapeutic substances *e.g.* dexamethasone. These legitimate therapeutic substances could also alter performance of the horse, so their use temporarily puts horses out of competition. These rules are applied in many jurisdictions, *e.g.* members of FEI and IFHA (FEI, 2017a; IFHA, 2017a). In Sweden, it is currently also a legal requirement that horses are kept out of competition when performance is altered by legitimate drugs (SJVFS 2013:43). Improvements in analytical techniques have made it possible to detect legal substances for an extended time post-administration and after environmental contamination, even at concentrations considered non-efficacious, so a zero tolerance policy is not an option for these drugs (Barragry, 2006). It is important that trainers/owners can trust that appropriate use of legitimate drugs will never lead to doping accusations. The use of quantitative PK and PD can be useful in establishing screening limits, detection times and withdrawal times which are all tools guaranteeing that correct use of legitimate drugs will not be considered as doping. Thus, the control of legitimate medication is upheld simultaneously as it allows veterinarians to apply good veterinary practice when treating sport-horses.

1.3.2 Screening limits

A screening limit (SL), also called screening sensitivity limit, is a concentration in a biological fluid (usually plasma or urine) that is considered irrelevant (Toutain, 2010b). The SL is decided by the regulatory authorities in the sport and is commonly confidential. However, one aim of the European Horserace Scientific and Liaison Committee (EHSLC) is to harmonise SL values between its members' jurisdictions. One commonly used method to decide a SL is a PK/PD approach often referred to as the Toutain model (Toutain & Lassourd, 2002). In brief, the approach involves calculating an effective plasma concentration (EPC) by means of the standard dose (*e.g.* as indicated in the marketing authorisation for the drug) and the clearance value (taken from the literature). A safety (uncertainty) factor (SF) is then applied to account for the variation in pharmacological parameters (*e.g.* clearance and drug potency values) between individuals and an irrelevant plasma concentration (IPC) is decided. The SF used must ensure that the IPC is truly irrelevant, *i.e.* the drug does not have any effect that alters the performance of the horse at this concentration. If the steady state ratio between plasma and urine is known, the Toutain model can

also be used to calculate effective and irrelevant urine concentrations (EUC and IUC).

1.3.3 Detection times

Both EHSLC and FEI have listed detection times (DT) for several therapeutic drugs (EHSLC, 2015; FEI, 2017b). A DT is based on an exposure study and represents the longest time a drug can be detected in plasma or urine after administration, *i.e*. the time point for the first negative sample, defined as when the sample concentration in all horses in the study is below a proposed screening sensitivity limit. The establishment of a DT follows a strict protocol. The dose and the route of administration must be as recommended by the manufacturer or represent most common clinical practice and the analytical procedure must be validated. The study uses healthy horses under scientific conditions which may not be representative to the conditions of a sport horse. Therefore, even though DT values are published by racing authorities, they should not be regarded as guaranteeing a negative doping test on race day. They only serve as one tool for the stakeholders, trainers and veterinarians to estimate a 'safe' time from treatment to competition.

1.3.4 Withdrawal times

A withdrawal time (WT) is the time from administration of a drug until the concentration in a biological fluid is below a proposed screening sensitivity limit in the entire population of horses (EHSLC, 2012). To minimise the risk of a positive sample on race day, WT must be longer than DT, which reflects the precise conditions of the exposure study. To compensate for possible variations in disposition of a drug within a population of horses (*e.g.* age, breed, sex, drug accumulation *etc*.) an adequate safety margin must be added to the DT. If any condition (dosing, pharmaceutical preparation, administrative route *etc.*) is different from the study that established the DT, this must also be taken into account. It has been proposed that variation in intrinsic factors (clearance, volume of distribution plasma-urine ratio *etc*.) is more important than variation in extrinsic factors (dose, route of administration *etc*.) (Toutain, 2010a). However, both intrinsic and extrinsic factors contribute to how the drug is dispositioned in body fluids. For drugs showing low variability in PK parameter estimates *e.g.* clearance, an increase of approximately 40% in DT might be a suitable WT, encompassing 90 % of the horse population, whilst doubling of the DT might be necessary with high variability in PK parameters (Toutain, 2010b; Toutain, 2010a; Tobin *et al.*, 2013)

1.3.5 Science as a screening limit additive for dexamethasone

There are some prerequisites for establishment of screening limits in plasma (and urine). Three basic assumptions according to Toutain & Lassourd (2002) are: i) The drug-induced responses are reversibly driven by the plasma concentration and a relationship between overall drug exposure and drug response exists ii) the dosing regimens currently used (under authority approval or in clinical practice) are appropriate, the doses are efficacious and does not greatly exceed the dose that gives 50 % of the maximum response, and iii) the drug disposition is linear within the range of plasma concentrations and plasma clearance is a parameter relating the dose to the plasma concentration.

The disposition of dexamethasone in plasma has been investigated in horses and there is consistent evidence of linear kinetics within the concentration ranges studied (Toutain *et al*., 1984; Cunningham *et al*., 1996; Soma *et al*., 2005; Grady *et al*., 2010; Soma *et al*., 2013). As already mentioned there are only very sparse quantitative PD data reported in the literature. Quantitative reports on plasma dexamethasone concentration-response time courses would improve the scientific evidence and be beneficial for anti-doping control of therapeutic substances in horse racing and equestrian sport.

1.4 Problem formulation

Dexamethasone plasma concentration has been proposed irrelevant for the response. The focus in this thesis has been to apply quantitative methods (*e.g.* nonlinear regression modelling) to experimental data in order to obtain quantitative information to use in the design of future experiments, for dose-calculations and in the control of therapeutic substances within the anti-doping control of equine athletes. This was done by means of assessing i) the dexamethasone exposure in plasma, urine and synovial fluid and ii) the biomarker response. Then i) and ii) was combined.

2 Aims

The overall aim of this thesis was to provide information about dexamethasone exposure and response in Standardbreds by means of quantitative methods. Specific aims were to:

- Determine the dexamethasone exposure in plasma, urine and synovial fluid after intramuscular, intravenous or intraarticular administration of dexamethasone-21-isonicotinate or dexamethasone sodium phosphate.
- Determine the relationships between i) dexamethasone plasma concentration and cortisol response and ii) the dexamethasone synovial fluid concentration and interleukine-1β response and lameness response in a lipopolysaccharide-challenged joint
- Determine clinical endpoint response to dexamethasone treatment of the LPS-challenged joint
- Position the quantitative information in an anti-doping perspective

3 Overview and progression of studies

The goal of this thesis was to investigate the exposure and response to dexamethasone. Overview and progression of studies are summarised in Table 1.

Table 1. *Thesis overview including problem formulation, hypothesis, key findings, conclusions and future perspectives*

	Paper I	Paper II	Paper III	Paper IV
Problem	Drug exposure and response in horses	Drug exposure and response Baseline variability	Drug exposure IL-1β response Cortisol response	Clinical endpoint response
Hypothesis	Concentration-response relationship possible	Improve model fitting	Concentration-response relationship	Clinical endpoint *vs* IL-1β response Concentration-response relationship
Key findings	Concentration-time course Response-time course Concentration-response hysteresis	Concentration-time course Response-time course Baseline variability	Concentration-time courses Concentration-IL-1β response Challenge variability	Challenge variability Concentration-lameness response relationship
Conclusions	Response is related to concentration Exposure: anti-doping perspective	The model mimicked data well Parameter consistency Improved test design	The model predicted drug exposure The model predicted IL-1β and cortisol response	Effect at doses lower than authorised Response duration Potency : Lameness~IL-1β
Future perspectives	Baseline of the response Increase parameter precision	Anti -inflammatory response	Clinical endpoint response	Model- and biomarker-based glucocorticoid screening

4 Material and Methods

4.1 Animals and ethics

Standardbred horses were used in all experiments. In Paper I, a study population consisting of six geldings aged 3-16 years and weighing 420-545 kg was used. In Paper II, a study population consisting of four mares and two geldings aged 6-20 years and weighing 430-584 kg was used. In Papers III and IV, a study population of three mares and three geldings aged 3-9 years and weighing 429-550 kg was used.

During experimental periods, the horses were kept in individual boxes and fed hay. Water was available *ad libitum*. In Paper I, the horses were also fed oats, while in Papers III and IV the horses were also fed concentrate (Champion komplett, Felleskjøpet,Lillestrøm, Norway). In Papers I and II the horses were kept in their home environment and allowed on pasture or in paddocks during daytime. In Paper III and IV the horses were brought into an animal hospital three days prior to the start of the each experimental period and kept in individual boxes during experimental periods. Between experimental periods the horses were kept on pasture. The horses were not exercised in any study.

The experiments described in Papers I and II were approved by Ethics Committee for Animal Experiments, Uppsala, Sweden (C232/8, C333/11). The experiment described in Papers III and IV was approved by the Norwegian Animal Research Authority (Forsøksdyrutvalget 2013/61618-1).

4.2 Experimental design, drug administration and sampling protocol

In Paper I dexamethasone-21-isonicotinate (Vorenvet®Vet 1 mg·mL^{-1} Boehringer Ingelheim Vetmedica, Malmö, Sweden) was administered as a single im dose of 0.03 mg·kg^{-1} in the neck. Blood samples were drawn at hours -120, -96,

-72, -48, -24, 0 (pre-dose), 1, 2, 4, 8, 12 and 24. Thereafter, blood was sampled at 24 hours interval up to 30 days. Urine was sampled before drug administration, twice within 24-hours post drug administration and thereafter once daily up to 30 days.

In Papers II-IV dexamethasone 21-phosphate disodium salt (DSP, Dexadreson 2 $mg \cdot mL^{-1}$, Intervet AB, Stockholm, Sweden) and 0.9 % saline (Natriumklorid Fresenius Kabi, Fresenius Kabi AB, Uppsala, Sweden) were used.

In Paper II the horses were divided into pairs and assigned to a randomised crossover design experiment including four treatments. Each treatment started with an intravenous (iv) bolus dose immediately followed by 3 h of constant-rate infusion. The dose levels were (bolus + infusion) 0.1 + 0.07 $\mu g \cdot kg^{-1}$, 1 + 0.7 $\mu g \cdot kg^{-1}$ and 10 + 7 $\mu g \cdot kg^{-1}$ dexamethasone. For the control 0.9 % saline was used. The concentrations of the dosing solutions were adjusted to give an infusion volume flow rate of 0.2 $mL \cdot kg \cdot h^{-1}$. Infusions were given using a volumetric infusion pump (Colleague, Baxter Healthcare Corporation, Deerfield, Illinois, USA). All dosing solutions were prepared within 30 min of administration by diluting DSP in saline. During control treatment horses received the same volume of saline solution as their respective dexamethasone treatment. Before the bolus dose (time = 0), a pre-dose blood sample was collected. Additional blood samples were drawn during and after infusion at hours 1, 2, 3, 4, 5, 6, 9, 12, 18, 24, 36 and 48. A minimum of a one-week wash-out period was allowed between drug treatments.

In Papers III and IV the experiment had a prospective, randomized and blinded cross-over design, with two treatments. An overview of the experimental set up is shown in Figure 1. In each treatment inflammation was induced into the antebrachiocarpal joint by means of LPS from Sigma-Aldrich (*E Coli* 055:B5). The LPS was diluted with 0.9% saline to give a concentration of 1 $ng \cdot mL^{-1}$. A total volume of 2 mL of LPS solution was injected into the joint at time 0. Two hours after LPS injection, either 2 mL dexamethasone solution (DSP diluted in 0.9 % saline) or an equivalent volume of control solution (0.9 % saline) were injected into the joint. The six horses used in the studies received individual doses dexamethasone. The dose levels were 0.01, 0.03, 0.1, 0.3, 1 and 3 mg per joint. A minimum of a three weeks wash-out period was applied between treatments. Synovial fluid and blood samples were collected at 0 (pre-LPS), 2, 4, 6, 10, 24, 28, 32, 48, 52, 56, 72, 76 hours. Three additional blood samples were drawn at 5, 20 and 40 minutes after dexamethasone/saline injection.

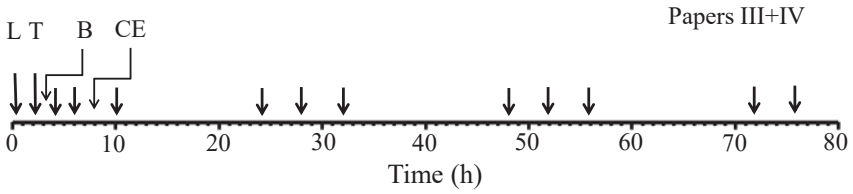

Figure 1. Overview of the time points of injections, sampling and clinical endpoint data collection (arrows) in paper III and IV. A total volume of 2 mL containing 2 ng lipopolysaccharides was injected in the antebrachiocarpal joint at 0h (L). At 2 h, dexamethasone sodium phosphate or saline was injected in the joint in a cross over design consisting of 2 treatments (T). Three extra blood-samples were collected 5, 20 and 40 minutes after dexamethasone or saline injections (B). Clinical endpoints were recorded at one extra time point 8 h after LPS administration (CE).

In Paper IV, data collection on clinical endpoints (CE) was as follows: The CE baseline was recorded three times on the day before LPS challenge. Various CE were also recorded before collection of synovial fluid and at one additional occasion eight hours after LPS administration. The CE were: rectal temperature (RT) measured by means of a digital thermometer, local skin temperature (ST) measured by means of a digital infrared thermometer (Fluke 574 cf, SR Automation AS, Asker, Norway) and joint circumference (JC) measured using a measuring-tape. Lameness was evaluated in trot (8 x 20 m in a straight line on flat concrete indoors). Lameness was subjectively scored by experienced clinicians by means of the American Association of Equine Practitioners (AAEP) lameness scale and objectively scored by means of an inertial sensor system-based method (Lameness Locator®, Equinosis, St. Louis, Missouri, USA). Before lameness evaluation one single axis accelerometer was attached to the bridle, one single axis accelerometer was attached between the right and left *Tuber sacrale* and one single axis accelerometer was attached to the dorsal aspect of the proximal phalanx of the right forelimb. Lameness was considered to be present when the minimum head height difference in vertical movement (MHH) between the stand phase of the right and left forelimb was greater than or equal to 7 mm and the average score for the study occasion was greater than its corresponding standard deviation. Non-lame Lameness Locator® (LL) scores were considered consistent with baseline data.

4.3 Analytical methods

4.3.1 Dexamethasone in plasma, urine and synovial fluid (paper I, II and III) and cortisol (hydrocortisone) in plasma (paper I and II)

Dexamethasone concentrations in plasma, urine and synovial fluid and cortisol plasma concentrations were determined with the use of Ultra High

Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS). Dexamethasone reference compound was acquired from Toronto Research Chemicals (North York, ON, Canada) and the internal standard $^{2}H_{4}$-dexamethasone (dexamethasone-d4) was purchased from CDN Isotopes through QMX Laboratories Ltd. (Essex, UK). Hydrocortisone and $^{2}H_{4}$-hydrocortisone (hydrocortisone-d4) were bought from Sigma-Aldrich (S:t Louis, MO, USA). The water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade or better and used without further purification. The Lower Limit of Quantification (LLOQ) for dexamethasone was 0.025 ng·mL^{-1} in plasma, 0.15 ng·mL^{-1} in urine and 0.38 ng·mL^{-1} in synovial fluid. The precision, expressed as the relative standard deviation (RSD), in the results for quality control samples in plasma was 3.0-11.8% for dexamethasone and 0.5-9.7 % for hydrocortisone while for dexamethasone in urine it was 3.1-13.3 % and in synovial fluid 2.7-7.8 %. The analytical method used for analyses in plasma and urine is described in detail in Paper I and that used for analyses in synovial fluid in Paper III.

4.3.2 Cortisol analyses in plasma (paper III)

For cortisol analyses a commercial Enzyme Linked Immunosorbent Assay (ELISA) was used (Cortisol ELISA (DE1887), DEMEDITEC, Kiel, Germany). This ELISA is intended for human plasma but the analysis has previously been described and validated for equine plasma (Boman, 2013). The optical density was measured at 450 nm using a microtitere plate reader (Wallac Victor², 1420 Multilabel Counter, Wallac Sverige AB). The cortisol concentrations were calculated by fitting a function to the optical densities by means of four parameter logistic fit using free online software from myAssays.com. The inter- and intra-test variation expressed as RSD, was 4 % and 11 % respectively.

4.3.3 Interleukine-1β analyses in synovial fluid (paper III)

For IL-1β analyses, a commercial ELISA (Equine IL-1β VetSet, Kingfisher Biotech, St. Paul, MN, USA) was used following the manufacturer's instructions. The optical density was measured and concentrations calculated as described for cortisol. To confirm assay usefulness in sample matrix, synovial fluid was spiked to 10 and 50 ng·mL^{-1}. Spiked samples were applied in duplicates and quantified to 8.45 and 10.70 for the 10 ng·mL^{-1} samples and 50.85 and 59.05 for the 50 ng·mL^{-1} samples. To assess inter-assay variability one sample was analysed on 8 occasions. To assess intra-assay variability one sample was analysed 9 times on 1 occasion.The inter- and intra-assay precision expressed as RSD was 30 % and 15 %, respectively.

4.4 Pharmacokinetic and pharmacodynamic concepts

Experimental dexamethasone data were described using compartment models in the respective studies. The predicted dexamethasone-time courses then served as constants 'driving' the PD models (Figure 2)

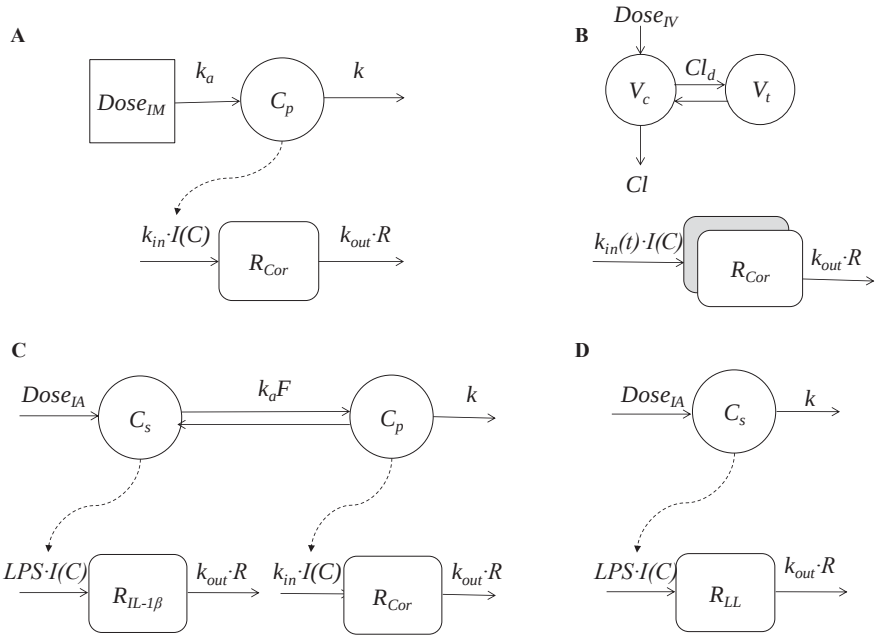

Figure 2. Schematic illustration of the disposition, drug mechanism function and response models used in Paper I (A), Paper II (B), Paper III (C) and Paper IV (D). Dose is the dose injected, C_p and C_s, are the dexamethasone concentration in plasma and synovial fluid, respectively; k_a and k are the absorption rate constant (from muscle to plasma) and the elimination rate constant of dexamethasone, respectively; and V_c,V_t,Cl_d and Cl are the volume of the central and peripheral compartment, the distributional parameter and the clearance respectively. In disposition from synovial fluid to plasma k_aF is the absorption rate constant with the bioavailability nested; k_{in} and k_{out} are the turnover rate and the fractional turnover rate of response; $I(C)$ is the drug mechanism function; LPS is the challenge function; R_{cor} is the cortisol response, $R_{IL\text{-}1\beta}$ is the IL-1β response and R_{LL} is the lameness response. In Paper II (B) one transit compartment (shaded) was used to capture the delayed onset of response.

4.4.1 Exposure of dexamethasone in plasma

In Paper I, a model including first order uptake and first order elimination was fitted to experimental dexamethasone concentration-time data. Exposure of dexamethasone in plasma (C_p) was described as

$$C_p = A \cdot [e^{-k\cdot t} - e^{-k_a\cdot t}] \quad (1)$$

where A is a pharmacokinetic macro parameter and k_a, k are the rate constants of the initial and terminal phase, respectively. The half-life of the terminal phase ($t_{1/2z}$) was calculated as

$$t_{1/2z} = \frac{ln(2)}{k} \quad (2)$$

A two compartment model was simultaneously fitted to the dexamethasone concentration-time data from all dose levels in Paper II. The disposition of dexamethasone in the central compartment was described as

$$V_c \cdot \frac{dC_p}{d_t} = Inf - Cl \cdot C_p + Cl_d \cdot C_t - Cl_d \cdot C_p \quad (3)$$

where C_t denote the dexamethasone concentration in the peripheral compartment, V_c is the central volume of distribution, Inf is the dose infused, Cl is the clearance of dexamethasone and Cl_d is the inter-compartmental distribution. The peripheral compartment was described as

$$V_t \cdot \frac{dC_t}{dt} = Cl_d \cdot C_p - Cl_d \cdot C_t \quad (4)$$

where V_t is the peripheral volume of distribution. The effective half-life in plasma was calculated as

$$t_{1/2} = \ln(2) \frac{V_{ss}}{Cl} \quad (5)$$

where V_{ss} (=V_c + V_t) denotes the apparent volume of distribution at steady-state.

In Paper III the input function of the two compartment model was adjusted to fit input to plasma from synovial fluid. The input function was described as

$$Input = C_S \cdot k_a F \quad (6)$$

Where C_s is the dexamethasone synovial fluid concentration and k_aF is the absorption rate constant with the bioavailability nested within the parameter. Hence, disposition of dexamethasone in the central compartment was described as

$$V_c \cdot \frac{dC_p}{d_t} = Input - Cl \cdot C_p + Cl_d \cdot C_t - Cl_d \cdot C_p \quad (7)$$

The disposition of dexamethasone in the peripheral compartment was described with Equation 4.

4.4.2 Exposure of dexamethasone in synovial fluid

Dexamethasone exposure in synovia following the 0.01, 0.03, 0.1 and 0.3 mg dose was described as

$$C_s = Dose \cdot A \cdot e^{-k \cdot t} \quad (8)$$

where C_s is the dexamethasone concentration in synovia, t is the time (in hours) and A and k are a pharmacokinetic macro parameter and the elimination rate constant respectively. Dexamethasone exposure in synovia following the 1 and 3 mg doses was described as

$$C_s = Dose \cdot A \cdot e^{-\alpha \cdot t} + Dose \cdot B \cdot e^{-\beta \cdot t} \quad (9)$$

where A and B, are the pharmacokinetic macro parameters and α and β the initial and the terminal slope factors, respectively.

4.4.3 Cortisol response

Cortisol turnover rate is inhibited by dexamethasone. The inhibitory drug mechanism function $I(C_p)$ is described by:

$$I(C_p) = 1 - \frac{I_{max} \cdot C_p^n}{IC_{50}^n + C_p^n} \quad (10)$$

where I_{max}, IC_{50} and n are the maximum drug-induced inhibition, the dexamethasone plasma concentration at 50% of drug-induced suppression of cortisol and the sigmoidicity factor respectively (Hill, 1910; Wagner, 1968). In Paper I, the turnover of cortisol response, R, was described as

$$\frac{dR}{dt} = k_{in} - k_{out} \cdot R \quad (11)$$

where dR/dt, k_{in} and k_{out} are the rate of change in response over time, the turnover rate and the first-order fractional turnover rate, respectively (Rescigno & Segre, 1966; Dayneka et al., 1993). Baseline of response, R_0, was described as

$$R_0 = \frac{k_{in}}{k_{out}} \quad (12)$$

The turnover rate was implemented as a function of the baseline R_0 and fractional turnover rate k_{out}.

$$k_{in} = R_0 \cdot k_{out} \tag{13}$$

The drug mechanism function (Equation 10) was then incorporated into Equation 11 to give Equation 14

$$\frac{dR}{dt} = R_0 \cdot k_{out} \cdot [1 - \frac{I_{max} \cdot C_p^n}{IC_{50}^n + C_p^n}] - k_{out} \cdot R \tag{14}$$

In Papers II and III the circadian turnover rate $k_{in}(t)$ was approximated by a cosine function described as

$$k_{in}(t) = k_{mean} + \alpha_{circ} \cdot cos\left[\frac{2\pi}{24} \cdot (t - t_0)\right] \tag{15}$$

where k_{mean}, α_{circ}, t and t_0 are the mean input rate, amplitude, clock time within the 24 h cycle and peak time, respectively. The ratio $2 \cdot \pi/24$ converts the 24 h period into radians. In Paper II one transit compartment was used in the PD model. Hence, the turnover of cortisol with the drug mechanism function incorporated was described as

$$\begin{cases} \frac{dR_1}{dt} = k_{in}(t) \cdot [1 - \frac{I_{max} \cdot C_p^n}{IC_{50}^n + C_p^n}] - k_{out} \cdot R_1 \\ \frac{dR_2}{dt} = k_{out} \cdot (R_1 - R_2) \end{cases} \tag{16}$$

where dR_1/dt and dR_2/dt are the rate of change of response in respectively compartment 1 and 2 of the turnover model. R_1 and R_2 denote the cortisol response and cortisol mean in plasma. The transit compartment model was not used in paper III. Hence, the turnover of cortisol with the drug mechanism function incorporated was described as

$$\frac{dR}{dt} = k_{in}(t) \cdot [1 - \frac{I_{max} \cdot C_p^n}{IC_{50}^n + C_p^n}] - k_{out} \cdot R \tag{17}$$

4.4.4 Lipopolysaccharide (LPS)-challenge model

In Papers III and IV a time dependent stimulator function (S_{LPS}) acting on the input rate of biomarker response was used. This approach was recently reviewed by Gabrielsson *et al.* (2015) and described as

$$S_{LPS} = A_{LPS} \cdot k_{LPS} \cdot t \cdot e^{-k_{LPS} \cdot t} \quad (18)$$

where A_{LPS} and k_{LPS} represent the amplitude and the rate constant, respectively, of the stimulatory function due to the LPS-challenge.

4.4.5 Interleukin-1β and lameness response

The time course of the Interleukin-1β (IL-1β) response in synovial fluid (Paper III) and lameness response (Paper IV) as a result of the LPS-challenge was described as

$$\frac{dR}{dt} = S_{LPS} - k_{out} \cdot R \quad (19)$$

Dexamethasone was assumed to inhibit IL-1β syntheses and lameness. The inhibitory function for IL-1β was described as

$$I(C_s) = 1 - \frac{C_s}{IC_{50} + C_s} \quad (20)$$

The time course for IL-1 β response with the drug mechanism function incorporated is then described as

$$\frac{dR}{dt} = S_{LPS} \cdot [1 - \frac{C_s}{IC_{50} + C_s}] - k_{out} \cdot R \quad (21)$$

and the inhibitor function for lameness response was described as

$$I(C_s) = \frac{1}{1 + (A_{inhib} \cdot C_s)} \quad (22)$$

where A_{inhib} is a parameter relating change in drug concentration to change in response. The time course for lameness response with the drug mechanism function incorporated was then described as

$$\frac{dR}{dt} = S_{LPS}^{\gamma} \cdot [\frac{1}{1 + (A_{inhib} \cdot C_s)}] - k_{out} \cdot R \quad (23)$$

where γ is an exponent of the stimulatory challenge function. The concentration of dexamethasone ($C_{s,50}$) that causes 50 % suppression of lameness response was calculated as

$$C_{s,50} = \frac{1}{A_{inhib}} \quad (24)$$

The equilibrium concentration-response relationship at steady state for cortisol was described as

$$R_{ss} = R_0 \cdot (1 - \frac{I_{max} \cdot C_p^n}{IC_{50}^n + C_p^n}) \quad (25)$$

and for lameness response as

$$R_{ss} = \frac{S_{LPS}}{k_{out}} \cdot \frac{1}{1 + (A_{inhib} \cdot C_s)} \quad (26)$$

4.5 Statistical calculations

In Paper I, the change in cortisol response over time was compared against R_0 using one-way ANOVA. Dunnett's test was used for *post hoc* analyses. Statistic calculations in Paper I were performed using computer software (Minitab 16.2.2, Minitab Inc., State College, Pennsylvania, U.S.A.).

In Paper IV, the area under the curve from all clinical endpoints from baseline to last observation (AUC_0^{last}) was calculated by means of the trapezoidal method using WinNonlin 4.0.1. As baseline value (t=0), the mean of all baseline observations was used. The data were then subjected to statistical hypothesis testing by means of two-sided Wilcoxon Rank Sum test for paired data observations using the statistical software *R* version 3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria). Clinical endpoint data from all dexamethasone doses were pooled before analyses. Statistical significance was considered if the p-value was smaller than 0.05.

5 Results and Discussion

5.1 Exposure to dexamethasone (Papers I, II and III)

5.1.1 Dexamethasone-21-isonicitinate intramuscularly

In Paper I, 0.03 $mg \cdot kg^{-1}$ dexamethasone-21-isonicotinate was administered intramuscularly to six horses. The main reason for quantifying dexamethasone plasma and urine concentrations was to obtain information of the dexamethasone concentration-time profile in plasma and urine to be used as support when estimating DT for equine athletes. The main reason for modelling experimental plasma dexamethasone-time data was to generate concentration-time profiles that could serve as input to the inhibiting function of the PD model.

Exposure in plasma

In plasma, observed peak concentration of dexamethasone was (mean ± standard deviation) 0.61 ± 0.12 $ng \cdot mL^{-1}$. Maximum concentrations were observed between 12 and 24 hours post drug administration (Figure 3). Dexamethasone was quantifiable (LLOQ 0.025 $ng \cdot mL^{-1}$) in plasma for up to 13 days (mean ± standard deviation: 8.3 ± 2.9 days). There was a two-fold variation in absorption rate and three-fold variation in elimination rate of dexamethasone in plasma (Table 2). The variation in elimination rates is also reflected in the calculated terminal half-life ($t_{1/2z}$) for dexamethasone in plasma which ranged between 18.8- and 58.6 hours for the six horses. This variation in half-life was also obvious considering the variation in time between drug administration and time when plasma concentrations dropped below the LLOQ for the six horses, as seen in Figure 3.

The plasma dexamethasone concentration-time profiles obtained in Paper I were different from those in Paper II. The differences were not due solely to the obvious absence of an absorption phase after use of the iv route of administration in Paper II. The mean terminal half-life after im administration

was 39 hours. This can be compared with the median effective half-life of 2.4 hours after iv administration used in Paper II. The pharmaceutic preparation of dexamethasone-21-isonicotinate used in Paper I is a suspension due to its low water solubility and acts as a slow release formulation. The prolonged and variable terminal half-life values reflect the slow release into plasma from the injection site (Gibaldi & Perrier, 1982).

A one-compartment model was fitted to the experimental data. In Paper I, some quantitative information on the slow release formulation of the drug was provided (input and output rate). The maximum observed plasma concentration was lower and the terminal phase extended compared with the concentration-time profile obtained after im administration of dexamethasone sodium phosphate solution (Soma *et al*., 2013). However, the study presented in this thesis was not a complete PK study due to the fact that no iv data were obtained.

Table 2. *Model estimated and derived parameters of dexamethasone exposure in plasma after 0.03 mg·kg^{-1} dexamethasone-21-isonicotinate administered intramuscularly to six horses*

	Model parameters			Derived parameter
Horse	A (ng·mL^{-1})	k (h^{-1})	k_a (h^{-1})	$t_{1/2z}$ (h)
1	2.05	0.03	0.08	27.1
2	0.88	0.02	0.11	43.0
3	0.49	0.01	0.15	70.1
4	0.80	0.01	0.10	48.7
5	1.40	0.03	0.08	25.2
6	2.50	0.03	0.06	23.4
Mean ± SD	1.37 ± 0.81	0.02 ± 0.01	0.10 ± 0.03	38.7 ± 19.0
95% CI	0.52 - 2.22	0.01 - 0.03	0.06 - 0.13	18.8 - 58.6

A is a pharmacokinetic macro parameter, k and k_a are the elimination rate constant and the absorption rate constant and $t_{1/2z}$ is the terminal half-life.

Exposure in urine

In urine, observed peak concentration of dexamethasone was (mean ± standard deviation) 4.2 ± 0.9 ng·mL^{-1} and was observed between 22 and 36 hours post drug administration (Figure 3). Dexamethasone was quantifiable in urine (LLOQ 0.15 ng·mL^{-1}) for up to 14 days (mean ± standard deviation: 9.8 ± 3.1 days). Inspection of the curves in Figure 3 indicates that the dexamethasone concentration-time profiles in plasma and urine were similar. Urine concentrations peaked later and were in median 7-fold higher and more variable, both within and between individual horses. The variability in dexamethasone urine data was about 2- to 4-fold higher than that in dexamethasone plasma data.

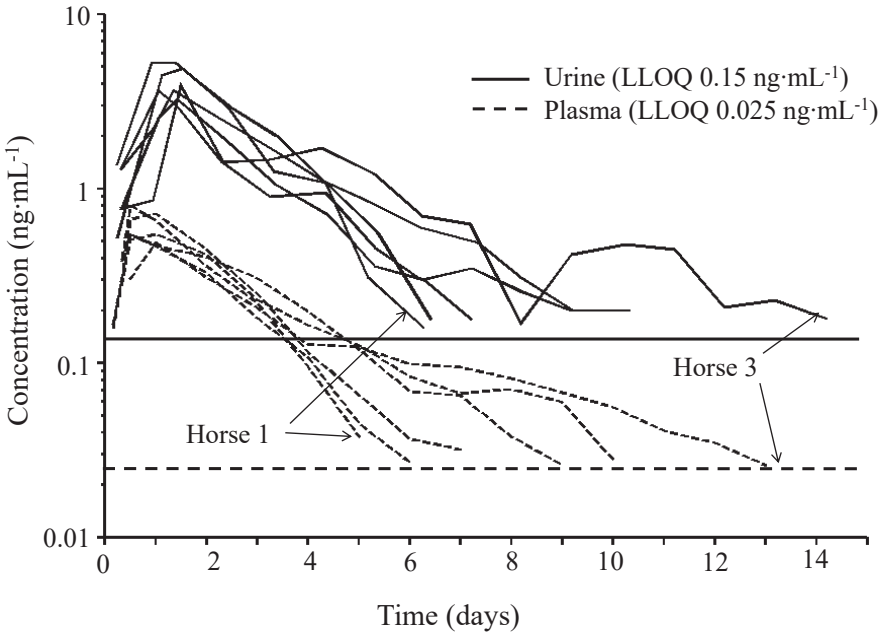

Figure 3. Semi-logarithmic plot of observed urine (solid lines) and plasma (dashed lines) dexamethasone concentration-time courses after 0.03 mg·kg^{-1} dexamethasone-21-isonicotinate administered intramuscularly to six horses. Horizontal lines denote the lower limit of quantification (LLOQ) in plasma (dashed line) and urine (solid line) respectively. The urine concentration-time course appears to be related to the plasma concentration-time course, which is illustrated by Horse 1 and Horse 3.

5.1.2 Dexamethasone-21-sodium phosphate salt intravenously

The main reason for quantifying and modelling dexamethasone plasma concentrations in Paper II was to characterise the disposition of dexamethasone in Standardbreds and to provide individual concentration-time profiles that could serve as input to PD analyses.

Dexamethasone disposition was linear within the studied concentration and time range and the concentration-time courses from all horses were similar (Figure 4). The two-compartment model accurately mimicked experimental data (Figure 5) and model parameters were estimated with good to acceptable precision. The parameter values were in median (range): 0.98 L·kg^{-1} (0.42-1.16), 0.82 L·kg^{-1} (0.53-1.11), 0.51 L·h^{-1}·kg^{-1} (0.46-0.56) and 0.2 L·h^{-1}·kg^{-1} (0.1-0.51) for V_c, V_t, Cl and Cl_d, respectively. The effective half-life, which is a measure of total removal of the drug from all compartments, was 2.4 h (2.0-2.6). Individual parameter estimates are given in Table 3. The parameters showed low variability between animals which is consistent with previous

findings in studies on Thoroughbreds (Cunningham *et al*., 1996; Soma *et al*., 2005; Grady *et al*., 2010; Soma *et al*., 2013).

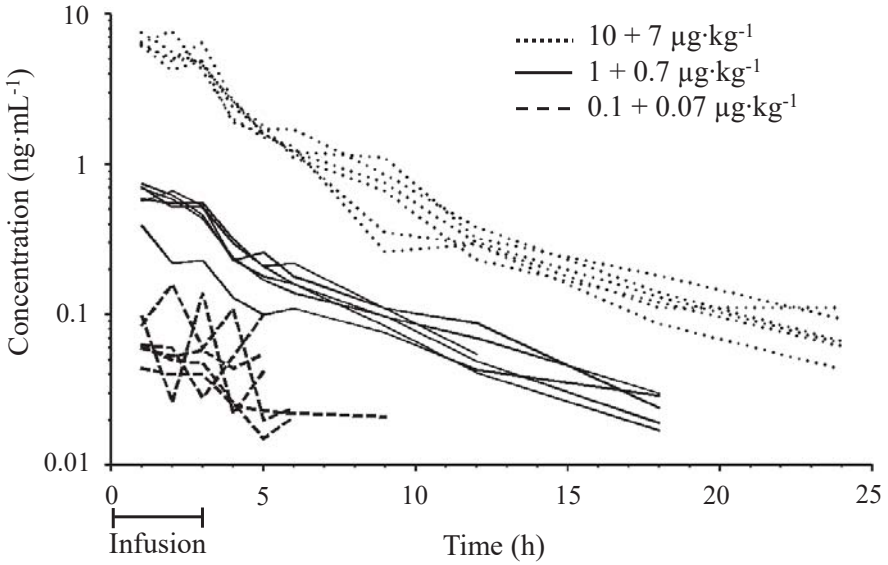

Figure 4. Semi-logarithmic plot of dexamethasone plasma concentration-time data during and after a bolus dose followed by three hours constant rate infusion of dexamethasone sodium phosphate to six horses. The dose levels were (bolus + constant rate infusion) 0.1 + 0.07 µg·kg^{-1}, 1 + 0.7 µg·kg^{-1}and 10 + 7 µg·kg^{-1}.

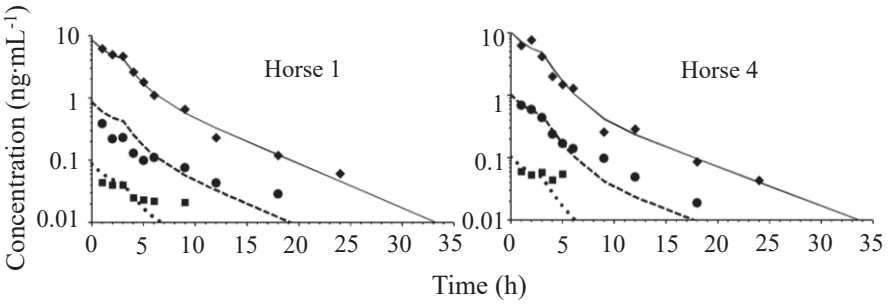

Figure 5. Semi-logarithmic plot of observed (symbols) and model predicted (lines) dexamethasone plasma concentration-time data for two horses given three different dose levels of dexamethasone. The dose levels were (bolus + three hours constant rate infusion regimen) 0.1 + 0.07 µg·kg^{-1} (filled squares, dotted lines), 1 + 0.7 µg·kg^{-1} (filled circles, dashed lines) and 10 + 7 µg·kg^{-1} (filled diamonds, solid lines).

Table 3. *Model estimated and derived pharmacokinetic parameters after a bolus dose followed by three hours of constant rate infusion of dexamethasone sodium phosphate administered intravenously to six horses.*

	Model parameters				Derived parameters	
Horse	V_c (L·kg^{-1})	V_t (L·kg^{-1})	Cl (L·h·kg^{-1})	Cl_d (L·h·kg^{-1})	V_{ss} (L·kg^{-1})	$t_{1/2}$ (h)
1	1.16	0.76	0.56	0.19	1.92	2.4
2	0.42	1.11	0.46	0.51	1.52	2.3
3	0.75	0.81	0.48	0.22	1.56	2.3
4	0.98	0.53	0.53	0.10	1.51	2.0
5	0.97	0.82	0.47	0.21	1.79	2.6
6	1.12	0.89	0.55	0.13	2.01	2.5
Range	0.42-1.16	0.53-1.11	0.46-0.56	0.10-0.51	1.51-2.01	2.0-2.6
Median	0.98	0.82	0.51	0.20	1.58	2.4

V_c and V_t are the central and the peripheral volume of distribution, Cl is the clearance, Cl_d is the inter-compartmental distribution, V_{ss} is the apparent volume of distribution at steady-state and $t_{1/2}$ is the effective half-life of dexamethasone.

5.1.3 Dexamethasone-21-sodium phosphate salt intraarticularly

Exposure in synovial fluid

Observed dexamethasone synovial fluid and plasma concentrations are shown in Figure 6. In synovial fluid collected after ia administration of dexamethasone in doses of 0.01, 0.03, 0.1 and 0.3 mg, dexamethasone was only quantified in concentrations above the LLOQ for synovial fluid (0.375 ng·mL^{-1}) up to 8 hours (three samples) after DSP injection. In synovial fluid collected after the 1 mg and 3 mg doses dexamethasone was quantified up to 30 hours (six samples) after DSP injection.

To experimental data from the 0.01, 0.03, 0.1 and 0.3 mg dose levels, a one-compartment model was fitted. To experimental data from the 1 and 3 mg doses, a two-compartment model was fitted. Observed and model predicted dexamethasone synovial fluid and plasma concentration-time courses are shown in Figure 7 for two of the horses. The sparse experimental data from the four lower doses only allowed fitting of a one-compartment model to the dataset. It is possible that higher doses DSP or a more sensitive analytical method than used in this work could have revealed a bi-phasic decline in the dexamethasone synovial fluid concentration-time course also in these horses. The model-estimated and derived PK parameters are shown in Tables 4 and 5. The area under the dexamethasone synovial fluid concentration-time curve (*AUC*) was 90, 550, 2 400, 7 600, 11 000 and 30 000 ng·mL^{-1}·h^{-1} for the 0.01,

0.03, 0.1 0.3, 1 and 3 mg doses respectively. The median (range) volume of synovial fluid cleared from dexamethasone was 71.4 $mL \cdot h^{-1}$ (39.5-113.2).

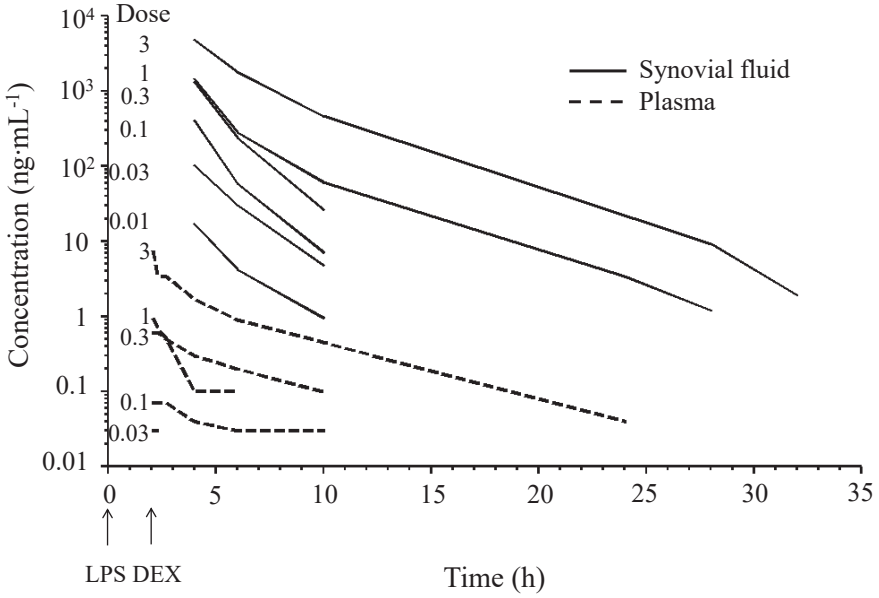

Figure 6. Spaghetti plot of observed synovial fluid (solid lines) and plasma (dashed lines) dexamethasone concentrations over time. Six horses were treated with different doses of dexamethasone sodium phosphate intraarticularly (DEX) two hours after intraarticular injection of 2 ng lipopolysaccharides (LPS). The doses were 0.01, 0.03, 0.1, 0.3, 1 and 3 mg dexamethasone. Dexamethasone was not quantifiable in plasma from the horse treated with 0.01 mg dexamethasone. The first synovial fluid sample was collected two hours after dexamethasone injection. The first plasma sample was collected 5 minutes after dexamethasone injection.

The terminal half-life ($t_{1/2z}$) for dexamethasone in inflamed synovial fluid ranged from 0.9 to 3.3 hours after ia administration of DSP. This is similar to the half-life in plasma after iv administration of DSP (Grady et al., 2010). A tentative stability control in the laboratory showed that the DSP ester is cleaved more rapidly in inflamed synovial fluid compared with synovial fluid from a healthy joint[1]. The inflammatory process cause increased blood flow and permeability over the vascular endothelium (Owen & Farrington, 1976). This might increase the distribution of dexamethasone into plasma. Conclusively, it is possible that the half-life in synovial fluid from a healthy joint would be longer. However, there is no dexamethasone half-life in synovial fluid from healthy joints reported, so this is pure speculation.

1. Mikael Hedeland Head of Department of Chemistry, Environment and Feed Hygiene National Veterinary Institute, e-mail 2014 04 01

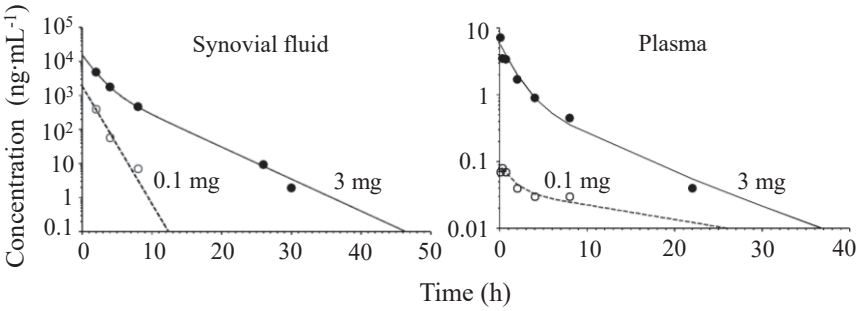

Figure 7. Observed (symbols) and model-predicted (lines) dexamethasone concentration-time courses in synovial fluid (left) and plasma (right) from two horses treated with individual doses (0.01 mg, empty circles, dashed lines and 3 mg, filled circles, solid lines) of dexamethasone injected into the antebrachiocarpal joint.

Table 4. *Model predicted and derived pharmacokinetic parameters describing exposure in synovial fluid following intraarticular administration of dexamethasone sodium phosphate to four horses. The parameters are estimated by means of a one-compartment pharmacokinetic model.*

	Model parameters		Derived parameter
Dose	A^1	k	$t_{1/2z}$
(mg)	(ng·mL^{-1})	(h^{-1})	(h)
0.01	50	0.54	1.3
0.03	300	0.55	1.3
0.1	1900	0.80	0.9
0.3	5700	0.76	0.9

A is a pharmacokinetic macro parameter, *k* is the elimination rate constant and $t_{1/2z}$ is the terminal half-life. [1] Parameter value reported with the dose incorporated

Table 5. *Model predicted and derived pharmacokinetic parameters describing exposure in synovial fluid following intraarticular administration of dexamethasone sodium phosphate to two horses. The parameters are estimated by means of a two-compartment pharmacokinetic model.*

	Model parameters				Derived parameters	
Dose	A^1	B^1	α	β	$t_{1/2\alpha}$	$t_{1/2\beta}$
(mg)	(ng·mL^{-1})	(ng·mL^{-1})	(h^{-1})	(h^{-1})	(h)	(h)
1	11000	310	1.07	0.21	0.6	3.4
3	13000	2400	0.70	0.22	1.0	3.2

A and *B* are pharmacokinetic macro parameters, α and β are the slope factors and $t_{1/2\alpha}$ and $t_{1/2\beta}$ the half-life of the initial and the terminal phase. [1] Parameter value reported with the dose incorporated

The dexamethasone synovial fluid-time courses after ia administration of 10 and 25 mg DSP were simulated using model parameters based on experimental data obtained from the 1 and the 3 mg doses (Figure 8). Increasing the dose

increased the time above the LLOQ for the analytical method. The terminal half-life of dexamethasone in synovial fluid is relatively short (0.9-3.4 h). Therefore, a relatively high increase in dose would probably produce a relatively small increase in duration of response.

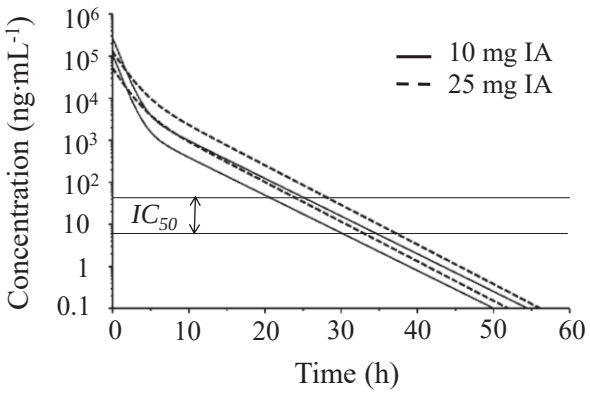

Figure 8. Semilogarithmic plot of the simulated dexamethasone synovial fluid concentration-time courses following intraarticular administration of 10 mg and 25 mg dexamethasone to two horses. Model parameters obtained from the 1 mg and 3 mg dose experimental data were used in simulations. Horizontal lines indicate the tentative potency (IC_{50}) range for IL-1β response.

Exposure in plasma

Dexamethasone was quantified (LLOQ 0.025 ng·mL^{-1}) in plasma from five horses (Figure 6). It was not possible to quantify dexamethasone in plasma from the horse injected with the lowest dose of dexamethasone (0.01 mg). Following the 0.03 mg dose, dexamethasone concentration was quantified in plasma in two samples. These samples were collected 5 and 20 minutes after DSP injection and the dexamethasone concentration in both was quantified to 0.03 ng·mL^{-1}. Following the 0.1, 0.3, 1 and 3 mg doses, dexamethasone concentrations was quantified in 5-7 samples for up to 24 hours. The dexamethasone plasma concentrations were consistently lower than the synovial fluid concentrations (Figure 6). Two hours after dexamethasone injection ia, synovial fluid concentrations were 3000-10000 fold higher than plasma concentrations. At the time of the last sampling in which dexamethasone concentration exceed the LLOQ in plasma, the synovial fluid concentrations were 200-1000 fold higher than the corresponding plasma concentrations. Maximum observed dexamethasone plasma concentration was observed within 40 minutes after dexamethasone injection into the joint. This together with the short terminal half-life (0.9-3.4 h) for dexamethasone in synovial fluid suggests rapid distribution from the joint into plasma after ia injection of DSP.

A two-compartment model with synovial fluid input to plasma was fitted to experimental dexamethasone plasma data for the four higher doses. The model

accurately mimicked experimental data (Figure 7). The main reason for modelling the plasma dexamethasone-time course after ia administration of DSP was to investigate the systemic exposure with respect to the cortisol response and to simulate the dexamethasone plasma concentration-time courses after higher doses DSP administered into the joint. Therefore, low parameter precision in the analyses was accepted. The parameter estimates were fixed and used as constants 'driving' the PD model and the simulations.

5.2 Response to dexamethasone exposure (Paper I-IV)

5.2.1 Cortisol response (Paper I, II and III)

Cortisol baseline

Cortisol plasma concentrations has often been used as a response biomarker to dexamethasone exposure in horses and other domestic species, *e.g.* dogs, cats and cattle (Toutain *et al.*, 1982; Toutain *et al.*, 1983; Toutain *et al.*, 1984; Smith & Feldman, 1987; Soma *et al.*, 2005; Abraham *et al.*, 2009; Grady *et al.*, 2010) The use of an integrative approach with quantitative methods in this thesis allowed separation of the drug properties (IC_{50}, I_{max} and n) from the physiologic system properties (k_{out} , t_0, α_{circ} and R_0). Quantitative analyses of cortisol response in Paper I was a secondary aim that mainly served to collect information as input to Paper II. The unaffected cortisol baseline in Papers II and III was characterised by a circadian variation with peak concentration in the morning and nadir concentrations in the afternoon/evening (Figure 9). In Paper I, data does not suggest circadian variation (Figure 10). This was due to the experimental protocol as all blood samples without dexamethasone present in the system were collected at 9 a.m. In addition, Paper I did not include any cortisol response data after control treatment with *e.g.* saline. As a consequence of the difference in experimental design and perhaps due to variation between study populations, the unaffected baseline of cortisol response appeared higher in Paper I compared to Papers II and III. The unaffected baseline range was 76-109 ng·mL^{-1} in Paper I, 34-57 ng·mL^{-1} in Paper II and 41-81 ng·mL^{-1} in Paper III. However, literature data indicate between study variations in cortisol baseline in horses that can be due to differences in study design and study populations (Toutain *et al.*, 1988b; Soma *et al.*, 2013). The results in those studies the results are consistent with the results from Paper II if the oscillation in cortisol baseline data is described by means of a cosine function. When the experimental design was similar to the design in Paper I, reported baseline data from the literature were also consistent with data given in Paper I.

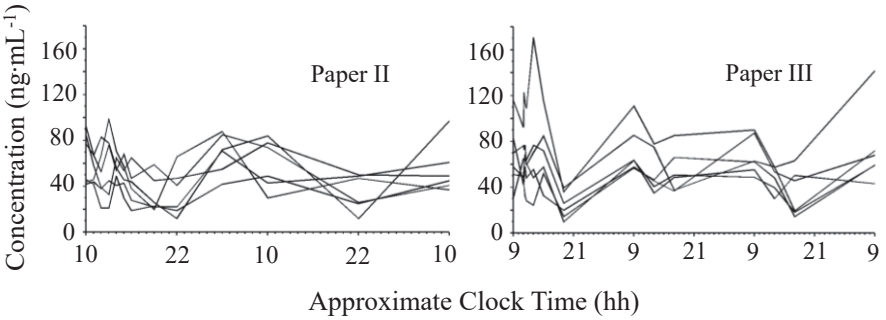

Figure 9. Spaghetti plot of observed cortisol-time courses during treatment with saline from six horses included in paper II (left) and six horses included in paper III (right).

Within animal variation in the cortisol baseline was observed. It is possible that external factors (stress or handling of the horses) stimulated cortisol release into plasma (Irvine & Alexander, 1994; Fazio *et al.*, 2008). Cortisol is episodically released into plasma which together with the relatively short half-life of the fractional cortisol turnover rate causes variation within the individual cortisol response-time courses (Toutain *et al.*, 1988a; Lassourd *et al.*, 1996). This might also explain the variation observed in this thesis.

The amplitude parameter (α_{circ}) was in median (range) 9.2 (6.8-24) in paper II and 16.2 (4.5-42.4) in paper III. Apart from physiology external factors, *e.g.* stress and handling of the horses also have an impact on this parameter (Irvine & Alexander, 1994; Fazio *et al*., 2008). It has been suggested that the circadian rhythm is easily obliterated in horses (Irvine & Alexander, 1994). In both Paper II and Paper III a circadian variation in the time-dependent cortisol response baseline (R_0) was observed. This finding suggests that it is possible that the variation in amplitude parameter reported in this thesis is mainly a result of physiological variations between the different horses studied and not a result of external factors.

To mimic the circadian variation of cortisol response a cosine function was used. Similar functions have been applied in PD models fitted to cortisol response data in humans (Lew & Jusko, 1993; Chakraborty et al., 1999). Median peak time of the cosine function (t_0) ranged from -2.8 to -4.7 and -0.2 to -5.8 in Paper II and Paper III, respectively. Start time for both experiments was between 9 and 10 a.m. Peak time of the circadian function in the morning was consistent with the literature (Hoffsis *et al.*, 1970; Bottoms *et al.*, 1972; Larsson *et al.*, 1979; Toutain *et al*., 1988b; Hart *et al.*, 2012; Bohak *et al.*, 2013). The fractional turnover rate (k_{out}) range was 0.16-0.71 h^{-1}, 0.47-1.5 h^{-1} and 0.14-1.24 h^{-1} in Paper I, II and III, respectively. The parameter varied 3- to 8- fold within studies and showed low variation between studies. The k_{out}

parameter gave a half-life of response ranging from 0.4 h to 4.2 h, which is consistent with values presented elsewhere (Lassourd *et al*., 1996; Soma *et al*., 2005; Soma *et al*., 2013).

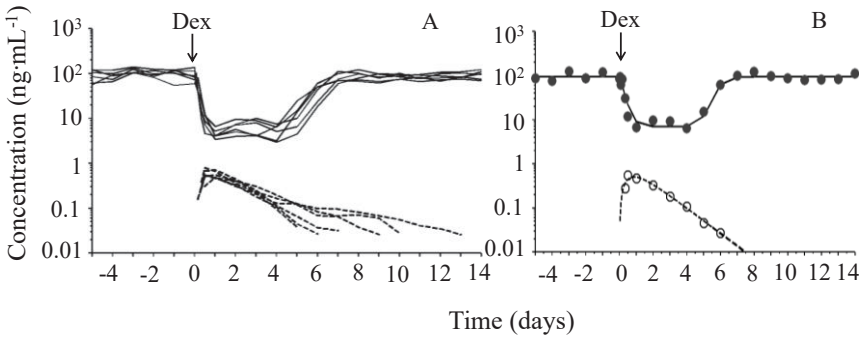

Figure 10. Semi-logarithmic plots of observed and model predicted cortisol and dexamethasone plasma concentration-time courses before and after intramuscular administration of 0.03 mg·kg^{-1} dexamethasone-21-isonicotinate (Dex) at time 0 (9 a.m.). Left plot (A): Spaghetti plot of observed cortisol-time courses (solid lines) and dexamethasone-time curses (dashed lines) in six horses. Right plot (B): Observed and model predicted cortisol-time courses (filled circles, solid lines) and dexamethasone-time courses (empty circles, dashed line) in one horse.

Cortisol response to dexamethasone exposure

In Papers I and II a clear dexamethasone concentration-cortisol response relationship was described and quantified in all horses (Figures 10, 11 and 12, Table 6). When dexamethasone became systemically available (*i.e.* in plasma) cortisol concentrations started to decrease. Cortisol response was suppressed until plasma dexamethasone concentration fell below its respective IC_{50}-value, whereupon cortisol response gradually returned to baseline. In Paper III, only data from the 1 mg and 3 mg doses DSP indicated response of cortisol to dexamethasone exposure (Figure 13). This was due to the low dexamethasone plasma exposure after ia injection of the lower doses. However, PD model parameters for those horses were consistent with parameters presented in Papers I and II. The PD model parameter estimates are shown in Table 6.

In Paper II, the time dependent baselines of cortisol response with dexamethasone present in the system were in median (range) 48 ng·mL^{-1} (33-65), 43 ng·mL^{-1} (27-66) and 35 ng·mL^{-1} (17-51) for the low, intermediate and high dose, respectively. In Paper III, the time-dependent baseline of cortisol response with the drug present in the system were 52 ng·mL^{-1} and 49 ng·mL^{-1} for data collected after ia administration of the 1 mg and 3 mg doses, respectively.

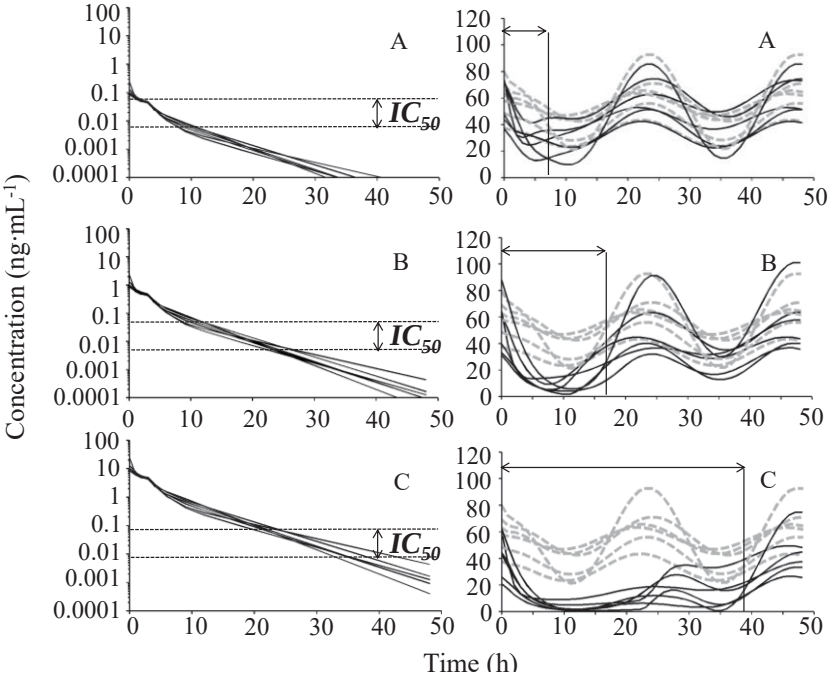

Figure 11. Left column: Predicted dexamethasone plasma-time courses after the low (A: 0.17 µg·kg^{-1}), intermediate (B: 1.7 µg·kg^{-1}) and high (C: 17 µg·kg^{-1}) doses of dexamethasone infused over 3 h. The horizontal dotted lines indicate the predicted potency (IC_{50}) range. Right column: Predicted cortisol response-time courses after the three concentrations of dexamethasone. The dashed grey lines denote the baseline-time course of the saline treatments. Arrows denote estimated duration of response.

The presence of dexamethasone in the system suppressed cortisol response in all horses in Papers I and II. In Paper III, suppression of cortisol response by dexamethasone could only be detected in horses treated with the 1 mg and 3 mg doses of dexamethasone. After the lower doses dexamethasone the systemic exposure of dexamethasone was insufficient to induce a level of suppression of cortisol response that could be detected using the current experimental protocol.

Dexamethasone is a potent (low IC_{50} value) and effective (high I_{max} value) suppressor of cortisol response in horses (Table 6). The potency value (IC_{50}-parameter) ranged from 25 to 132 pg·mL^{-1} in Paper I and from 6 to 65 pg·mL^{-1} in Paper II. In paper III the IC_{50}-value was 43 pg·mL^{-1} and 32 pg·mL^{-1} for the 1 mg dose and the 3 mg dose, respectively. The value of the I_{max} parameter ranged from 0.92-0.97 in Paper I and 0.77-0.97 in Paper II. In Paper III the I_{max} parameter was 0.77 for the 1 mg dose and 0.80 for the 3 mg dose. The *n* parameter gives the shape of the slope and is an indication of the sensitivity of

a physiological system. A high value of the *n*-parameter indicates a steep curve and an almost dichotomous concentration-response relationship. Consequently, at low concentrations a small increase in dexamethasone plasma concentration produces a large increase in cortisol response. At concentrations well above the IC_{50}-value, a relatively large increase in concentration produces a relatively small increase in response (Figure 14). The main consequence of increasing the dose further is an increase in the duration of response, but not the intensity of response (Figures 11 and 14).

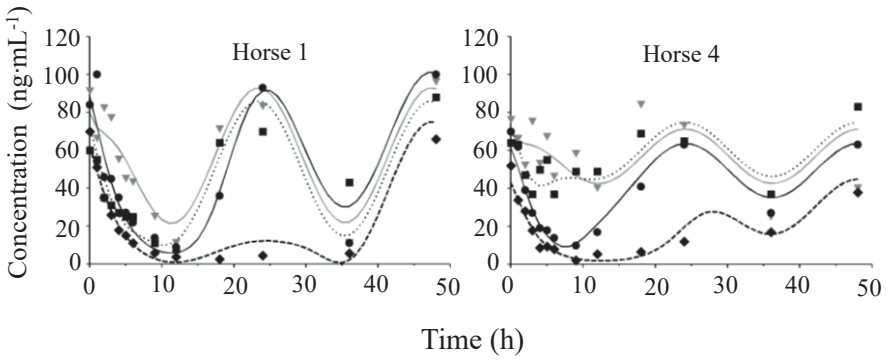

Figure 12. Observed (symbols) and model predicted (lines) cortisol plasma concentration-time courses from two horses given a 3 hour infusion of dexamethasone at three different dose levels. Horse 1 (left) shows high and horse 4 (right) low amplitude in cortisol oscillation. The dose levels were 0.17 $\mu g \cdot kg^{-1}$, (filled squares, dotted black lines), 1.7 $\mu g \cdot kg^{-1}$ (filled circles, solid lines) and 17 $\mu g \cdot kg^{-1}$ (filled diamonds, dashed lines). Baseline is represented by grey triangles, solid grey lines.

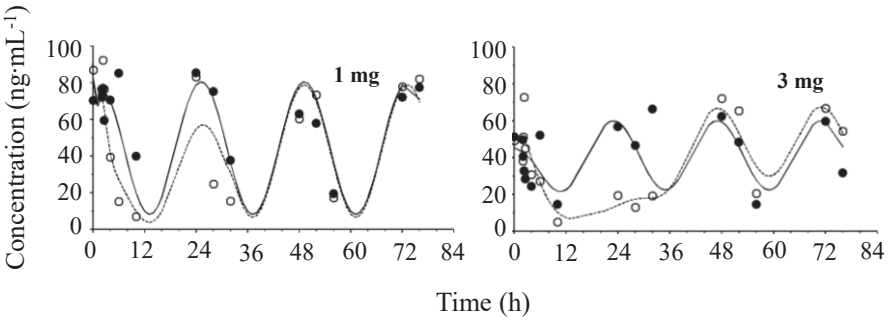

Figure 13. Observed (symbols) and model predicted (lines) cortisol plasma concentration-time courses from two horses injected with 1 mg (left) or 3 mg (right) dexamethasone (empty circles, dashed lines) or saline (filled circles, solid lines) intraarticularly at time=0.

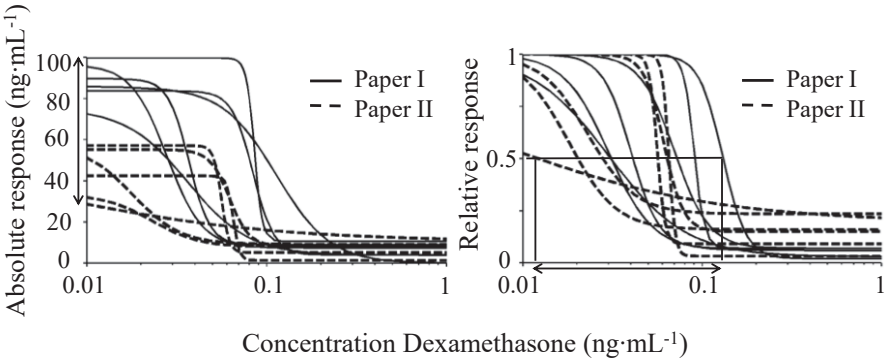

Figure 14. Semi-logarithmic plots of plasma dexamethasone concentration-cortisol response relationship from paper I (solid lines) and paper II (dashed lines). Left: Absolute response where the arrow denotes variation in cortisol response baseline. Right: same data as the left hand plot but on a relative response scale where the arrow denotes the variation in potency.

Clinical application of the quantitative cortisol response model

One application of the quantitative PD model described in Paper II was to predict the outcome of one testing protocol and drug scheduling in the dexamethasone suppression test (Dybdal et al., 1994). This test is used in medical investigations to diagnose hyperadrenocorticism. Simulated duration of cortisol response was more than 20 hours in healthy Standardbreds after iv administration of 20 $\mu g \cdot kg^{-1}$ dexamethasone (Figure 15). Cortisol response showed oscillating behaviour at maximum response. These oscillations at maximum drug effect were more pronounced in horses with larger amplitude (α_{circ}-parameter) in cortisol baseline oscillation than in horses with smaller α_{circ}-values. Cortisol response oscillated between 3-15 $ng \cdot mL^{-1}$ in the high amplitude horse in the simulation. These results persisted with increased dose (40 $\mu g \cdot kg^{-1}$). Horses with hyperadrenocorticism often suffer from hyperglycaemia and insulinaemia, which are also effects of dexamethasone (Keen et al., 2004; Tiley et al., 2007). Therefore, decreased doses of dexamethasone may lower the risk of additional unwanted effects due to high dexamethasone exposure. Duration of response after the iv dosage regimen of 20 $\mu g \cdot kg^{-1}$ was satisfactory for the test, as can be seen in Figure 15. A test protocol based on a single plasma sample was not optimal due to the oscillating behaviour of cortisol response around the proposed threshold. This problem can be solved by more frequent sampling since time-series sampling for a quantitative assessment of the cortisol response would improve the quality of the test results. Additional samples, *e.g.* at 7 and 22 hours after dexamethasone administration, would capture the fluctuations in response more adequately than a single time point evaluation. If a single time point sample is used the test

results should be interpreted with caution due to the oscillating behaviour of cortisol. Use of the iv compared with the im route of administration increases the control of plasma exposure otherwise confounded by within- and between-individual variations in rate and extent of absorption and allows a lower dosage regimen. It should be noted that the median parameter estimates were used in simulations in this study. Individual patients may deviate even more from the predicted cortisol-time course. Reported values from the dexamethasone suppression test may therefore be different from the cortisol response presented here. Hence, this example merely exemplifies the power of model-based analyses.

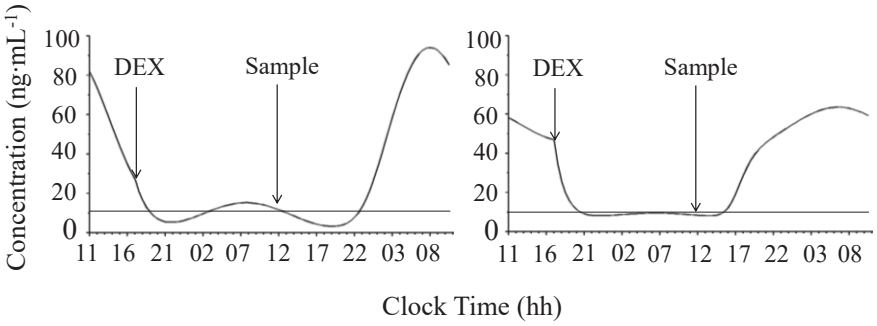

Figure 15. Simulated cortisol-time courses after a 20 $\mu g \cdot kg^{-1}$ bolus dose of dexamethasone administered intravenously to a high amplitude horse (left) and a low amplitude horse (right). Arrows indicate the proposed injection time (17 pm) and sampling time (19 h post dexamethasone administration) proposed by Dybdal *et al.* (1994) for the dexamethasone suppression test. The horizontal lines indicate the proposed plasma cortisol threshold in the dexamethasone suppression test (10 $ng \cdot mL^{-1}$).

Table 6. *Model predicted and derived pharmacodynamic parameters of cortisol response (papers I, II and III)*

Paper(s)	Horse	IC_{50} (pg·mL^{-1})	I_{max}	n	k_{out} (h^{-1})	$t_{1/2kout}$ (h)	t_0 (h)	α_{circ} (ng·mL^{-1})	R_0 (ng·mL^{-1})
I	I:1	33	0.97	2.0	0.25	2.8	-	-	-
I	I:2	87	0.93	14.9	0.31	2.3	-	-	-
I	I:3	132	0.98	6.6	0.18	3.9	-	-	-
I	I:4	74	0.93	4.5	0.71	1.0	-	-	-
I	I:5	35	0.93	5.5	0.16	4.4	-	-	-
I	I:6	25	0.94	3.5	0.33	2.1	-	-	-
II	II:1	18	0.84	4.0	0.56	1.2	-4.0	24	57
II	II:2	65	0.97	25	0.47	1.5	-4.7	8.5	42
II	II:3	62	0.85	8.0	0.74	0.9	-4.8	6.8	55
II	II:4	56	0.91	30	0.55	1.3	-3.2	9.9	57
II	II:5	23	0.77	3.0	0.69	1.0	-4.6	7.9	34
II	II:6	6	0.81	0.7	1.5	0.5	-2.8	17	55
III	III:1	-	-	-	1.24	0.6	-0.2	42	81
III	III:2	-	-	-	0.14	5.0	-4.5	4.5	47
III	III:3	-	-	-	0.61	1.1	-3.3	16.8	43
III	III:4	43	0.77	2.7	1.16	0.6	-0.5	43.2	45
III	III:5	32	0.80	4.1	0.43	1.6	-5.3	11	41
III	III:6	-	-	-	0.53	1.3	-5.8	16.8	57
I-III	Range	6-132	0.77-0.98	0.7-30	0.14-1.5	0.5-5.0	-5.8- -0.2	4.5-43.2	34-81
1-III	Median	39	0.92	4.1	0.54	1.3	-4.3	13.9	51

IC_{50}, I_{max} and n are the potency value, efficacy value and the sigmoidicity factor, respectively. k_{out} and $t_{1/2kout}$ is the fractional turnover rate and the half-life of response, t_0 is the peak time of the circadian function, α_{circ} is the amplitude, and R_0 is the utilized baselines of response.

5.2.2 IL-1β response (Paper III)

LPS-challenge and changes in IL-1β concentration over time

It was not possible to quantify IL-1β with the method used in this study in any of the pre-LPS administrations samples. During saline treatment, IL-1β was detected in concentrations above the assay LLOQ (3.125 ng·mL^{-1}) in synovial fluid samples from five horses collected between 2 and 10 hours after LPS administration (Table 7, Figure 16). Peak concentration during saline treatment was observed 4 hours after LPS administration and ranged between 10 ng·mL^{-1} and 51 ng·mL^{-1}. In the horse treated with 0.01 mg dexamethasone IL-1β was 3.2 and 5.0 at 4 and 6 hours after LPS administration, respectively. After treatment with LPS + saline in the same horse, IL-1β was not above the LLOQ in any samples. During dexamethasone treatment IL-1β was above the assay LLOQ in samples from the 0.01, the 0.1 and the 0.3 mg dose with observed peak concentration 4 to 6 hours after LPS injection. The IL-1 β concentration was higher after treatment with 0.01 and 0.3 mg dexamethasone compared to respective treatment with saline. The IL-1β concentrations were lower after treatment with dexamethasone in horses receiving 0.03, 0.1, 1 and 3 mg doses compared with treatment with saline.

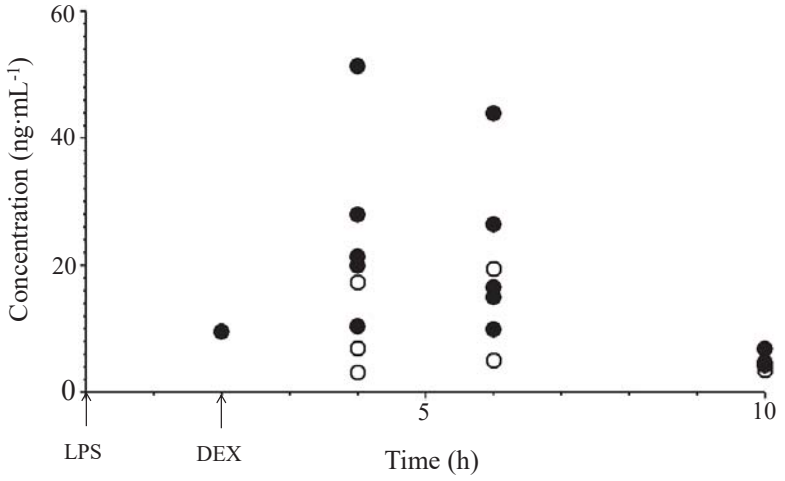

Figure 16. Observed synovial fluid IL-1β concentration-time courses in six horses after intra-articular administration of 2 ng lipopolysaccharides (LPS) at hour 0 and dexamethasone sodium phosphate (empty circles) or saline (filled circles) at hour 2. The horses were treated with one dose each. The doses were 0.01 mg, 0.03 mg, 0.1 mg, 0.3 mg, 1 mg and 3 mg dexamethasone.

Synthesis of IL-1β is inhibited by dexamethasone at transcriptional level (Lee *et al.*, 1988; Waterman *et al.*, 2006; Hirsch *et al.*, 2012). In addition, exposure to glucocorticoids decreases the stability of IL-1β mRNA and the release of IL-1β (Snyder & Unanue, 1982; Lee *et al*., 1988; Waterman *et al*., 2006). Samples from two horses (treated with 0.01 mg and 0.3 mg dexamethasone) were found to have higher synovial fluid IL-1β concentrations after saline treatment compared with dexamethasone treatment. The most likely explanation is a combination of variability in LPS-challenge response between treatments, the low doses of dexamethasone administered (0.01 mg and 0.3 mg), potential incomplete injections of LPS or DSP and intra-assay variation in the analytical method. The strong evidence for dexamethasone induced inhibition of IL-1β synthesis combined with the data set in this thesis led to that experimental data from horses treated with 0.03, 0.1, 1 and 3 mg dexamethasone and respective saline treatment were used in modelling.

Table 7. *Observed synovial fluid IL-1β (ng·mL^{-1}) concentrations before and after intraarticular administration of 2 ng lipopolysaccharides at time 0 and dexamethasone (Dex) or saline at 2 h. Six horses were used in a two-treatment cross over design experiment. The horses were treated with different doses of dexamethasone.*

	IL-1β (ng·mL^{-1})									
Dose	0h		2h		4h		6h		10h	
(mg)	Dex	Saline	Dex	Saline	Dex	Saline	Dex	Saline	Dex	Saline
0.01	<LLOQ	<LLOQ	<LLOQ	<LLOQ	3	<LLOQ	5	<LLOQ	<LLOQ	<LLOQ
0.03	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	51	<LLOQ	44	<LLOQ	5
0.1	<LLOQ	<LLOQ	<LLOQ	10	7	20	5	17	<LLOQ	<LLOQ
0.3	<LLOQ	<LLOQ	<LLOQ	<LLOQ	17	10	20	10	4	4
1	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	21	<LLOQ	15	<LLOQ	<LLOQ
3	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	28	<LLOQ	26	<LLOQ	7

Pharmacodynamic model

The sparse data set combined with lack of quantifiable IL-1β concentrations during treatment with 0.03, 1 and 3 mg dexamethasone made it impossible to fit the PD model to the individual IL-1β concentration-time courses. Therefore, data from the horses treated with 0.03, 0.1, 1 and 3 mg were modelled simultaneously (Figure 17). Data from each individual dexamethasone synovial fluid concentration-time course served as constants 'driving' the PD model fitted to the IL-1β experimental data. Hence, the variation in PD model parameter estimates does not reflect true between-individual variation. The model parameter estimates derived by simultaneously modelled IL-1β response

to these four dexamethasone-time courses were in median (range) 0.5 h^{-1} (0.49-0.51), 19 ng·mL^{-1} (7-45), 0.47 h^{-1} (0.47-0.47) and 51.9 ng·mL^{-1} (51.6-52.3) for k_{out}, IC_{50}, k_{LPS} and A_{LPS}, respectively.

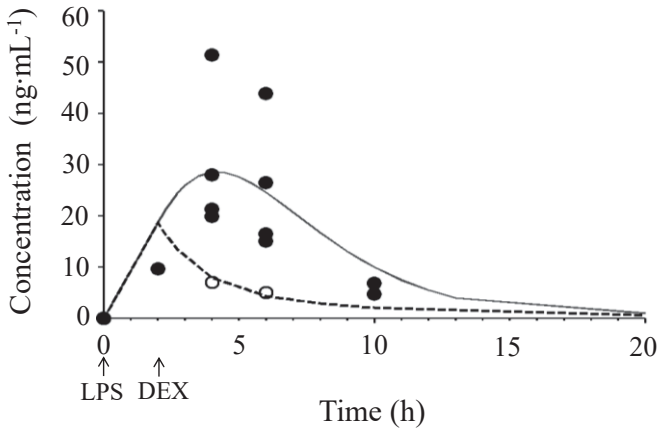

Figure 17. Observed (symbols) and model predicted (lines) IL-1β synovial fluid-time courses in four horses after intraarticular injection of 2 ng lipopolysaccharides (LPS) at hour 0 followed by dexamethasone sodium phosphate (empty circles, dashed line) or saline (filled circles, solid lines) at hour 2. The four horses were treated with one dose of dexamethasone each. The doses were 0.03, 0.1, 1, and 3 mg.

5.2.3 Clinical response (Paper IV)

In Paper IV the clinical endpoints JC (joint-circumference), ST (local skin temperature), RT (rectal temperature) and lameness, scored by both the AAEP (American association of equine practitioners)-score and LL (lameness locator)-score, were used as biomarkers of clinical response. There was no difference in clinical response baseline between the two treatment protocols (LPS+ dexamethasone and LPS + saline).

Lameness response

The LPS-challenge + saline treatment increased lameness response score measured as difference in MHH (minimum head height) using LL data compared with baseline (Figure 18). The LL-response AUC_0^{Last} was lower in all horses after treatment with dexamethasone compared to treatment with saline. The LL-score expressed as AUC_0^{Last} ranged from 150 to 2100 mm·h after treatment with saline and from 10 to 440 mm·h after treatment with dexamethasone The difference between the two treatment protocols using the LL-response scores was significant (P=0.03).

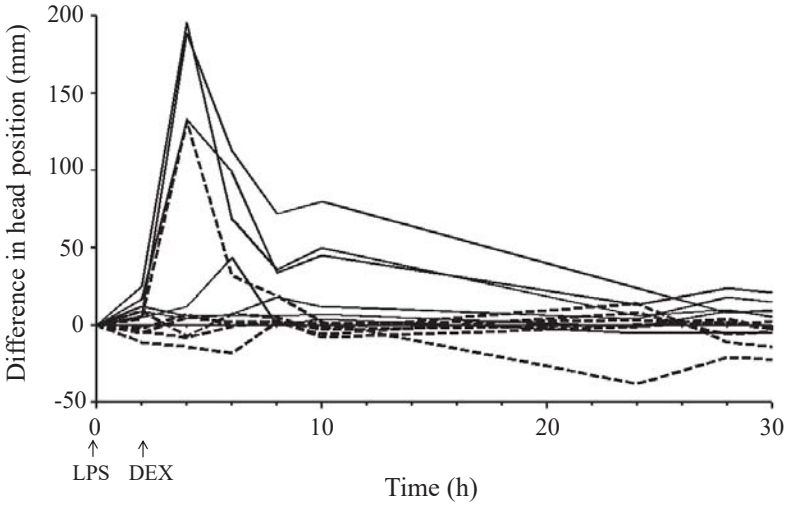

Figure 18. Spaghetti-plot of observed minimum head height relative to baseline scored by the lameness locator system in six horses challenged with 2 ng lipopolysaccharides (LPS) in the antebrachiocarpal joint at hour 0. At hour 2 either saline (solid lines) or dexamethasone (DEX, dashed lines) was injected in the LPS-challenged joint. The six horses received one dose of dexamethasone each. The doses were 0.01, 0.03, 0.1, 0.3, 1, and 3 mg.

The LPS-challenge + saline treatment increased lameness response score using AAEP data compared with baseline in five of the six horses. The AAEP data collected after LPS-challenge + saline treatment from horse #6 (which was treated with 0.01 mg dexamethasone as active treatment) did not indicate lameness. Lameness response score expressed as AAEP AUC_0^{Last} ranged from 1 to 30 degrees lameness·h after treatment with dexamethasone and from 0 to 80 degrees lameness·h after treatment with saline. The AAEP lameness response score AUC_0^{Last} was lower after treatment with 0.03-3 mg dexamethasone, but not after the 0.01 mg dose compared with saline treatment. The difference between the different treatment protocols using the AAEP scores was not significant (p=0.21). In this thesis, the LL-score was more sensitive in detecting differences in lameness than the AAEP-score. In the LL-scoring system, the measured outcome variable can range from 0 to (in theory) infinity. The AAEP scale ranges from 0 to 5. This and the fact that the AAEP lameness score AUC_0^{Last} for the horse treated with 0.01 mg dexamethasone was lower than the saline AUC_0^{Last} data from the same horse probably explains why lameness was significant using the LL, but not the AAEP score.

Inertial sensor-based systems *e.g.* LL, showed repeatability in a straight line as used in this thesis and are comparable with gait analyses by video recording (Keegan *et al.*, 2004; Keegan *et al.*, 2011). It has also been proposed that intra- and inter-rater variability may bias and limit the usefulness of subjective gait

analyses scales such as the AAEP score (Keegan *et al.*, 1998; Arkell *et al.*, 2006; Hewetson *et al.*, 2006). In Paper IV, LL was shown to be a more sensitive method to evaluate lameness than the AAEP scale. Paper IV also demonstrated between-animal MHH variation after LPS-challenge followed by saline injection (Figure 18). Incomplete ia injection might have contributed to the between-horse variation in response to the LPS-challenge and the dexa-methasone treatment between the horses, despite care being taken when injections were performed. Aspiration of synovial fluid followed by injection performed without resistance was used as confirmation of correct needle placement within the joint cavity. Lack of an objective confirmation method (*e.g.* ultrasonography) of needle placement is a weakness that should be considered in interpretation of the results. However, between-animal variation in lameness induced by comparable doses of LPS administered ia has been reported (Ishihara *et al.*, 2005; Santos *et al.*, 2009; van Loon *et al.*, 2013).

Joint circumference, skin temperature and rectal temperature

The LPS-challenge increased the JC response (p=0.03) and ST response (p=0.03) compared with the contralateral non-challenged joint (Figure 19). The increase in AUC_0^{Last} for the ST and JC response was larger after saline injection than after dexamethasone injection for all doses except for 0.01 mg. The difference between treatments was not significant (p=0.16 and 0.06 for ST and JC response, respectively). There was no difference in RT between the two treatments. No signs of septic arthritis were observed during the study or in the following days. In Paper IV, none of the variables ST, JC or AAEP score decreased significantly during treatment with LPS + dexamethasone compared with LPS + saline. However, in the work presented in the present thesis the AUC_0^{Last} parameter decreased for all three variables after ia dexamethasone (Figure 19). Despite that the differences between the two study protocols not were statistic significant, Figure 19 show a potential decrease in AAEP score, ST and JC after treatment with dexamethasone compared with treatment with saline. A likely explanation for the non-significant results is the low dexamethasone exposure following the lowest doses used combined with a small study population consisting of 6 horses. Consistent with Paper IV, significant lameness reduction but no significant decrease in JC after ia injections of glucocorticoids has been shown in horses (Kay *et al*., 2008).

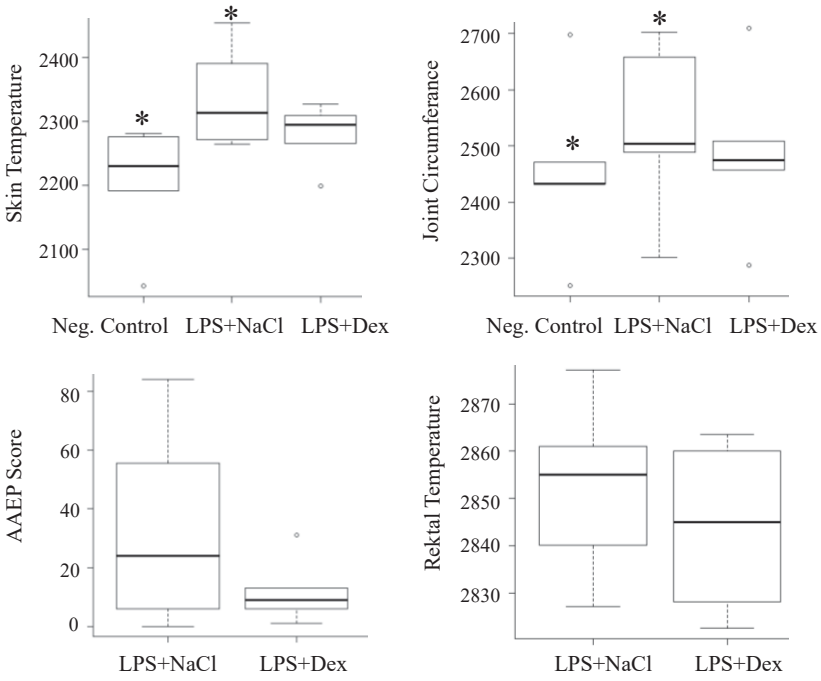

Figure 19. Boxplot showing the area under the curve from baseline to last observation (AUC_0^{Last}) for the clinical responses local skin temperature, joint circumference, AAEP lameness score and rectal temperature after lipopolysaccharide (LPS)-challenge treated with saline (NaCl) or dexamethasone (Dex) intraarticularly. In the upper plots, the negative control refers to the contralateral non-challenged joint. The box borders represent the first and the third quartile. The thick line represents the median and the whiskers represent 1.5 times the inter quartile range. Open circles represent observations not included within whisker range. *indicates statistically significant difference ($p<0.05$) between negative control and LPS + saline treatment.

Lameness response-time course

The observed LL response score data from all horses are shown in figure 18. The LPS-challenge + ia saline induced a difference in MHH in all horses compared with baseline. Median (range) MHH was 96 mm (12-196). The maximum MHH was observed within 8 hours. The MHH score then returned to baseline within 24 hours after LPS-challenge in five horses and within 32 hours in one horse. For one horse MHH score indicated lameness in the contralateral forelimb between hours 24-52 after LPS-challenge + 0.3 mg dexamethasone. In that horse, the AAEP-score indicated lameness from hour 48 to 72.

Pharmacodynamic model

A PD model was fitted to experimental lameness response data from five horses. It was not possible to fit the model to data from the horse treated with the lowest dose (0.01 mg). The observed and fitted lameness scores from two

horses are shown in Figure 20 and the simulated equilibrium steady state dexamethasone-lameness response relationship for two horses is shown in Figure 21. The model parameters were in median (range) 0.32 per hour (0.18-0.35), 3.3 mm (3.1-4.8) and 16 (8.2-43.6) for the k_{LPS}, A_{LPS} and γ-parameter, respectively. The corresponding value for the PD model parameter A_{inhib} 0.06 per concentration (0.0004-0.19) and the calculated concentration at which lameness is reduced by 50 % was 15 ng·mL^{-1} (5-2000). The variation in the amplitude parameter was lower compared to the 14-fold between-animal variation in MHH data. The decreased between-animal variation in amplitude was a result of the additional γ-parameter. The use of this parameter as an exponent in the LPS-challenge function was necessary to capture the rapid onset and peak observation of the challenge in the sparse experimental data.

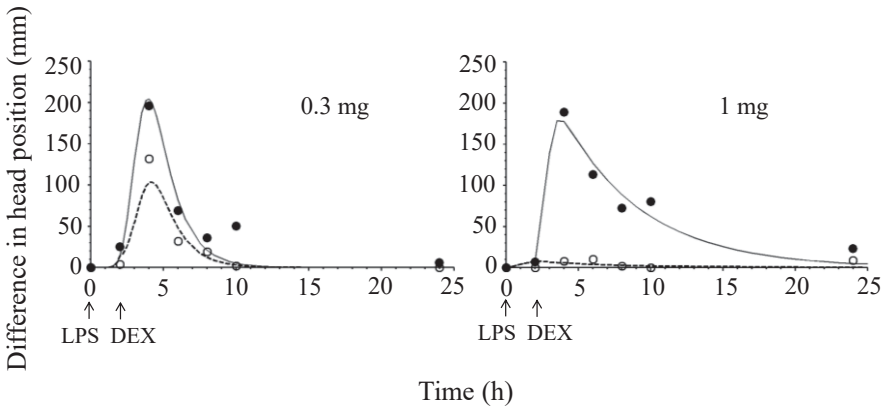

Figure 20. Observed (symbols) and model-predicted (lines) differences in minimum head position scored by the lameness locator system in two horses challenged with 2 ng lipopolysaccharides (LPS) in the carpal joint at hour 0. At hour 2, either saline (filled circles, solid lines) or dexamethasone (empty circles, dashed lines) was injected (DEX) in the LPS-challenged joint at a dose of 0.3 mg (left plot) and 3 mg (right plot).

Fitting a PD model to experimental LL response data provided input data to demonstrate the equilibrium dexamethasone synovial fluid concentration-response relationship (Figure 21). The PD model captured the onset, intensity and duration of both LPS-challenge and response to dexamethasone (Figure 20). Concentration values at 50 % suppression of the lameness response after dexamethasone treatment were derived. There was a 400-fold variation in derived concentrations at 50 % suppression of the lameness response, because the horse treated with 0.3 mg dexamethasone was an outlier. The high sensitivity to the LPS-challenge in combination with the relatively low dose dexamethasone resulted in high LL-response score after treatment with

dexamethasone in this horse compared with the other horses (Figure 20). Since the PD model only included one PD parameter (A_{inhib}) this resulted in low parameter value and high derived concentration values for 50 % suppression of lameness response. If it would have been possible, the use of a more complex PD model (*e.g.* I_{max}-model) might have adjusted for this. Then the efficacy parameter would have estimated the low intensity of response in this horse and the potency value probably lower. However, due to the sparse experimental data was it not possible to use those PD-models to estimate parameters with precision and low parameter correlation.

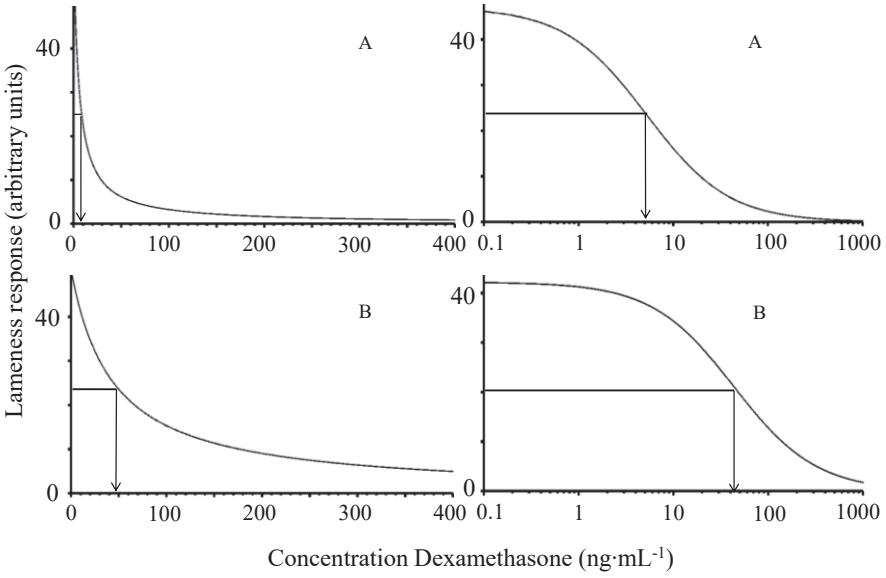

Figure 21. Simulated equilibrium dexamethasone synovial fluid concentration-lameness response relationship. The plots are based on pharmacodynamic lameness response data from paper IV. One horse where lameness was supressed with 50 % at lower dexamethasone synovial fluid concentration (upper plots A) and one horse where lameness was supressed with 50 % at higher dexamethasone synovial fluid concentration (lower plots, B). Arrows indicate the estimated concentration at where lameness was supressed with 50 %. The linear plots (left column) show that an equivalent change in concentration produced a larger change in response at lower concentrations compared with the higher concentrations. The semi-logarithmic plots (right column) highlight the concentration range for the largest change in response.

However, when PD data from the horse treated with 0.3 mg dexamethasone were excluded, the concentration at which lameness was supressed by 50 % showed a 9-fold variation (5-45 ng·mL^{-1}) between the four remaining horses. The 9-fold variation is consistent with previously reported variation in potency values within a study population (Toutain *et al.*, 2001) The range 5-45 ng·mL^{-1}

is also consistent with the tentative potency value for IL-1β response (19 $ng \cdot mL^{-1}$) in synovial fluid in this thesis. The LL-response to LPS-challenge followed by saline treatment returned to baseline within 32 hours in all horses. Therefore, a maximum of seven observations were made after each treatment. In addition, LPS-challenge + dexamethasone treatment data indicate full inhibition of LL response in five horses. Consequently, it was not possible to fit a more extended PD model (*e.g.* sigmoid I_{max}-model) to the data-set and obtain model- estimated parameters. The fit of a less complex model to data in order to mimic the LL-response-time course was successful in five horses but with relatively low precision of the A_{inhib}-parameter. Hence, the parameter should be interpreted with caution and the tentative quantitative information given in Paper IV should primarily be used as input to future experiments. The dexamethasone synovial fluid concentration at which lameness is reduced by 50 % can also be used in anti-doping methodology for control of therapeutic substances.

Interestingly enough, the horse treated with 0.03 mg dexamethasone became lame on the opposite non-challenged/non-treated leg after treatment with dexamethasone (Figure 22). Neither LL response score nor AAEP response score indicated lameness for this horse during base-line. However, it was denoted "asymmetrical" in trot. One possible explanation might be that the horse had similar pain in both front limbs. The treatment with dexamethasone then produced an anti-inflammatory response that decreased lameness in the treated joint and lameness on the contralateral leg became detectable.

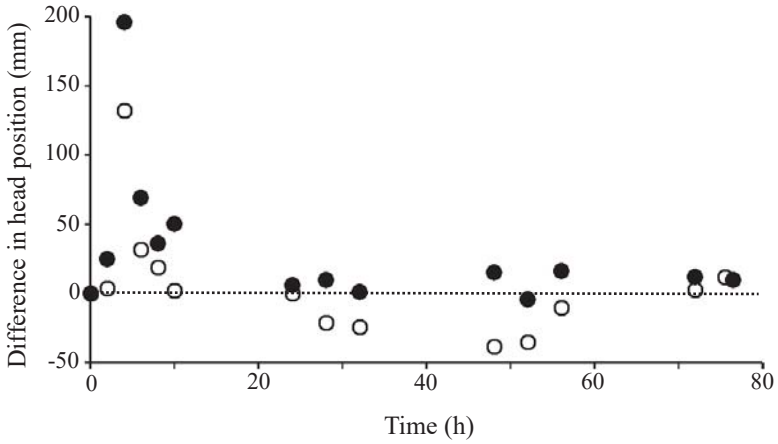

Figure 22. Observed difference in minimum head position over time in one horse treated with lipopolysaccharides (LPS) + saline (filled circles) and LPS + 0.3 mg dexamethasone (empty circles) intraarticularly. Negative values indicate lameness on the contralateral leg.

6 General Discussion

6.1 Cortisol response and clinical response in a biomarker perspective

6.1.1 General biomarker considerations

Biomarkers can be used for instance to predict the clinical outcome of drug treatment. One classification of biomarkers used in quantitative pharmacology aims at placing the biomarker somewhere on the causal path between phenotype and response (Peck *et al.*, 2003; Danhof *et al.*, 2005; Rolan *et al.*, 2007). Danhof *et al.* (2005) presented a classification of biomarkers with seven different categories namely phenotype/genotype, drug concentration, target occupancy, target activation, physiological response, patho-physiological response and clinical response (which are clinical endpoints rather than biomarkers *per se*). Using this classification and quantitative information on the biomarkers might increase the precision in prediction between species and from experimental to clinical studies.

6.1.2 Cortisol from a biomarker perspective

It is unlikely that cortisol response can be considered on the causal path between dexamethasone administration and the anti-inflammatory response. However, if quantitative PD data on cortisol response are consistent with biomarkers on the causal pathway to anti-inflammatory response, cortisol response could be an option for screening other glucocorticoid substances. The quantitative information could then be used in the anti-doping control methodology of therapeutic substances in racing and equestrian sports. Unfortunately, the data presented here did not include any suitable biomarker in the healthy horse. During the experimental work in Paper II, samples were collected to evaluate gene (mRNA) expression and quantification in plasma of

e.g. IL-1β, IL-10 and Annexin A1. Unfortunately, the quality and amount of mRNA in the samples were insufficient for further analyses. Moreover it was not possible to quantify cytokines or Annexin A1 due to insufficient sensitivity of the analytical assays (data not shown). Nevertheless, cortisol response might be a useful biomarker for purposes other than anti-inflammatory response screening. In Papers I and II a clear plasma dexamethasone concentration-cortisol response relationship was shown. It is reasonable to believe that intra cellular concentration of dexamethasone is more important for the response since the glucocorticoid receptor are mainly located within the cell (Baxter & Tomkins, 1971; Levinson *et al.*, 1972). It is also reasonable to believe that there is equilibrium in steady state between concentration in plasma and intra-cellular concentration since no active transport of dexamethasone over the cell membrane has been reported. Consequently, dexamethasone plasma exposure profiles might be relevant for use as surrogate concentrations in PD analyses of the response to dexamethasone. The median potency-value for cortisol response based on the data presented in this thesis is 39 $pg \cdot mL^{-1}$. In comparison, the potency-values for IL-1β response and lameness response presented in Papers III and IV were approximately 1000-fold larger. This indicates that cortisol response is a sensitive biomarker of systemic exposure to dexamethasone in horses. This is consistent with reports that cortisol response in humans is more sensitive to glucocorticoid plasma exposure than lymphocyte response and neutrophil response (Mager *et al.*, 2003; Stark *et al.*, 2006).

6.1.3 Example on a clinical application of quantitative information

The quantitative model-based analysis of cortisol response was also used to evaluate and suggest improvement in the predictability of one dexamethasone suppression test protocol originally presented by Dybdal *et al.* (1994). The results presented in Paper II (Figure 15) suggest that a protocol based on a single sample might be misleading when interpreting the test result. Hence, additional samples are recommended to increase the predictability of the test.

6.1.4 Lipopolysaccharide-challenge model from a biomarker perspective

Intraarticular LPS injection in challenge tests has been widely used in horses. In doses up to 2.5 ng the LPS-challenge induced local inflammation and produced *e.g.* lameness response, JC response, ST response and synovial fluid cytokine response (Palmer & Bertone, 1994; Easter *et al.*, 2000; Campebell *et al.*, 2004; de Grauw *et al.*, 2009; van Loon *et al.*, 2010; Ross *et al.*, 2012). At this LPS dose level, the horses continued to bear weight on the treated leg and no clinical relevant signs on systemic variables (*e.g.* fever) have been reported.

The lameness response duration is 24-48 hours. LPS doses at 25 ng or higher increased the intensity and duration of the LPS-challenge and at those dose-levels the horses are unwilling to bear weight on the LPS-challenged leg, while the challenge also affects systemic variables, *e.g.* increased rectal temperature and heart rate (Hawkins *et al.*, 1993; Palmer & Bertone, 1994; Lindegaard *et al.*, 2010).

In Paper IV, there was no difference in rectal temperature between the two treatment protocols or between base-line and LPS + saline treatment. All horses maintained their appetite throughout the time range studied. The horses were willing to be led by hand (both in walk and in trot). The IL-1β response and lameness response during treatment with LPS + saline was characterised by rapid onset, short duration and relatively rapid return to baseline. Release of another pro inflammatory cytokine, tumour necrosis factor alpha, induced by LPS administered ia in horses or iv in other species show similar time courses, but with shorter duration (Campebell *et al*., 2004; Wang *et al*., 2007; Gabrielsson *et al*., 2015). However, the results presented in this thesis are consistent with reported lameness duration induced by comparable LPS doses administered ia to horses (Palmer & Bertone, 1994; Easter *et al*., 2000; Santos *et al*., 2009). During treatment with LPS + dexamethasone IL-1β response and lameness response were lower than treatment with LPS + saline.

The challenge test in this thesis was designed to mimic a clinical situation. Therefore, the LPS-challenge was administered two hours before the dexamethasone or saline treatment instead of injecting the drug before the challenge which is another challenge test design (Salomone *et al.*, 1998; Primiano *et al.*, 2016). The modelling of biomarker response data after LPS-challenge was performed using a model with a time-dependent stimulator function acting on the input rate of biomarker response. This approach has recently been reviewed (Gabrielsson *et al*., 2015). An alternative option would have been a model with discontinuous stimulatory functions allowing biomarker formation to take place only during a defined period (Gozzi *et al*., 1999; Chakraborty et al., 2005; Wyska, 2010; Sukumaran *et al*., 2012). However, the use of a continuous stimulatory function might mimic the physiological response more adequately and probably allow a more biologically relevant description of the biomarker-time course. The model accurately mimicked experimental data (Figures 17 and 20). Experimental data on the biomarker response-time courses and the unaffected biomarker baseline-time course were modelled simultaneously in Papers II, III and IV (the data-set in Paper I did not include any control data). Simultaneous fitting of the model to data from all dose-levels allowed the model to use all available information in the data-set during estimation of the model parameters and increased the

parameter precision (Draper & Smith, 1998; Gabrielsson & Weiner, 2016). In light of the sparse data-set and the different doses used in Papers III and IV, the model-predicted biomarker response-time courses and the parameter estimates seem reasonable. The consistency across the studies using two different models suggests that the concentration range where lameness is supressed by 50 % lies somewhere within the true potency range of the studied population, despite the low parameter precision.

6.2 Dexamethasone in a medication perspective

6.2.1 Response to intraarticular glucocorticoid injections

In Paper IV, ia dexamethasone was shown to reduce lameness response induced by LPS-challenge of the equine joint at doses lower than the approved dosses (Medical Products Agency, 2015). It has been clearly shown in both experimental and clinical studies that glucocorticoid therapy at approved doses or doses commonly used by clinicians reduces clinical signs of joint disease (Trussel, 1965; Van Pelt & Riley, 1967; Houdeshell, 1969, 1970; Van Pelt *et al*., 1970; Van Pelt *et al*., 1971; Vernimb *et al*., 1977; Salles-Gomes *et al*., 2003; Kristiansen & Kold, 2007; Labens *et al*., 2007; Kay *et al*., 2008; Brommer *et al*., 2012; de Grauw *et al*., 2016; Knych *et al*., 2017b). In those studies, no synovial fluid glucocorticoid concentrations were quantitatively related to the biomarker response and few doses were evaluated. The response to ia treatment with dexamethasone has been explored in the dose range 6-12 mg per joint (Salles-Gomes *et al*., 2003; Brommer *et al*., 2012). Those studies did not include any control treatment (*e.g.* saline).

6.2.2 Glucocorticoid dosing from a safety perspective

The efficacy of dexamethasone at the dose levels used in this thesis is important. It has been proposed that glucocorticoid exposure has a detrimental effect on equine cartilage. Under non-inflammatory conditions such studies have comprised both *in vivo* and *in vitro* experiments across various biomarkers for cartilage turnover and including various glucocorticoids (Chunekamrai *et al.*, 1989; Trotter *et al.*, 1991; Saari *et al.*, 1992; Todhunter *et al.*, 1998; Robion *et al.*, 2001; Murray *et al.*, 2002; Celeste *et al.*, 2005). As biomarkers for unwanted effects on cartilage, proteoglycan synthesis, collagen syntheses and glycosaminoglycan synthesis have been used, among others. The potentially detrimental effects appear to increase with increased glucocorticoid exposure (Jolly *et al.*, 1995; Todhunter *et al.*, 1996; Murphy *et al.*, 2000; Fubini *et al.*, 2001; Frean *et al.*, 2002; Richardson & Dodge, 2003; Doyle *et al.*, 2005). Inflammatory conditions are also potentially detrimental for the

equine joint and glucocorticoid exposure appears to partly reverse these effects (Frisbie *et al.*, 1997; MacLeod *et al.*, 1998; Todhunter *et al.*, 1998; Bolt *et al.*, 2008). One proposed explanation is that matrix breakdown during inflammatory processes in the joint due to increased enzymatic activity, for instance by cyclooxygenases, matrix metalloproteinases and aggrecanase, which are inhibited by glucocorticoids (Tung *et al.*, 2002; Busschers *et al.*, 2010; Garvican *et al.*, 2010).

Combined with the demonstrated dose-response relationship the quantitative input is beneficial. Based on the model parameters the dexamethasone synovial fluid concentration-response relationship (R_{ss}) was simulated in this thesis. The simulation results indicated that a small change in concentration within the concentration range around the potency value produced a relatively large change in response (Figure 21). At synovial fluid concentrations obviously higher than the potency value, a large change in concentration produced a relatively small change in response.

6.2.3 Lower doses from a safety perspective

Lower doses might increase the number of injections needed to maintain drug exposure over time. Moreover, there are case reports describing septic arthritis after ia injections in horses (Lapointe *et al.*, 1992). During the experimental work in this thesis, a total of 156 joint punctures were performed. No signs of septic arthritis were detected during the experiment or in the following days. This result suggests that the risk of infection is low when aseptic injection techniques are used. This is also supported by a recent report suggesting that infection is uncommon following joint injections (Steel *et al.*, 2013). Nevertheless, septic arthritis is a severe condition and the risk should not be neglected. The use of a local route of administration without puncture of the joint would eliminate the risk of septic arthritis. Iontophoresis can transport dexamethasone across the skin and in theory deliver dexamethasone into synovial fluid. The technique has shown to be effective compared with placebo for knee pain in humans (Li *et al.*, 1996; Paturi *et al.*, 2010). Unfortunately the observed maximum dexamethasone concentration after iontophoresis is lower than 2.3 ng·mL^{-1} synovial fluid (Kaneps *et al.*, 2002). This can be compared to the concentration range 5-45 ng·mL^{-1} (outlier data not included) at 50 % suppression of LL response presented in this thesis. For now, joint puncture appears to be the only option for treating osteoarthritis locally with dexamethasone.

6.2.4 Future perspectives and animal welfare considerations

The use of a sustained release formulation has the potential to reduce both the free glucocorticoid concentration in synovial fluid and the number of injections. The terminal half- life in synovial fluid is 3 days (2-7), 0.8 days (0.6-4) and 0.5 days after ia administration of methylprednisolone acetate, triamcinolone acetate and isoflupredone acetate, respectively (Knych *et al.*, 2013; Knych *et al.*, 2014; Knych *et al.*, 2016). The initial and terminal synovial fluid half-lives after ia administration of betamethasone phosphate/acetate is 0.5 and 8 days respectively (Knych *et al.*, 2017a). A treatment protocol with documented response (effect) that also minimises the risk of detrimental effects in the joint and lowers the risk for septic arthritis improve animal welfare. Data from exposure studies (*e.g.* Knytch *et al.*, 2014) combined with future studies of the glucocorticoid synovial fluid concentration-response relationship are also needed to estimate the duration of response after ia injection of other glucocorticoid substances. This quantitative information would improve existing treatment protocols and anti-doping control of therapeutic substances in equine athletes, which is important for animal welfare.

6.3 Dexamethasone in an anti-doping perspective

To ensure the animal welfare of equine athletes, they must be given accurate medical treatment if they suffer disease or injury. To protect the integrity of the sport and the animal welfare, horses are not allowed to participate in competition during medical treatment. To minimise the risk of drug-traces at irrelevant concentrations after legitimate administrations to horses being detected in doping controls and falsely lead to doping accusations, DT, WT and SL are used by the regulatory authorities and practising veterinarians (Wong & Wan, 2014).

6.3.1 Screening Limits

Toutain & Lassourd (2002) introduced a model to decide SL based on plasma concentrations after administration of a dose of a drug that is assumed to produce therapeutic plasma concentrations. In Paper I, the exposure of dexamethasone in plasma an urine after injection of 0.03 $mg \cdot kg^{-1}$ dexamethasone-21-isonicotinate im were described. This dose was decided based on an assumed horse weight of 500 kg and the approved volume 15 ml Vorenvet vet. (1 $mg \cdot mL^{-1}$) as described in the summary of product characteristics (Medical Products Agency, 2010). Hence, the observed plasma concentration was assumed relevant for calculation of irrelevant plasma concentration of dexamethasone. The observed maximum dexamethasone

plasma concentration ranged from 0.5 ng·mL^{-1} to 0.8 ng·mL^{-1}. A safety factor (SF) of 500 as proposed by Toutain & Lassourd (2002) applied on plasma data suggests the SL 1 pg·mL^{-1} plasma. Unfortunately, there are no PD plasma data available relating the anti-inflammatory response to plasma concentration of dexamethasone. Compared with the IC_{50}-range for cortisol response (6-132 pg·mL^{-1} plasma) the irrelevant plasma concentration appeared appropriate. Applying a SF of 500 to the lowest proposed synovial fluid concentration reducing the lameness response by 50 % reported in Paper IV (5 ng·mL^{-1}) gives a value of 10 pg·mL^{-1}. This is not a proposed irrelevant concentration or SL. More quantitative data are needed to establish a therapeutic concentration based on scientific data. However, it confirms the need for low SL for dexamethasone as proposed in this thesis. It is possible to use a lower SF if there is quantitative PK and PD information about the drug (Toutain & Lassourd, 2002). Since cortisol response cannot be assumed to be on the causal pathway between dexamethasone exposure and anti-inflammatory response future quantitative studies on other biomarkers are warranted. Inadequate quantitative PD information increases uncertainty and consequently the size of the SF needed in calculations of IPC. Until scientific data support the use of a smaller SF than used in this thesis for calculation of IPC, a low SL as proposed in this thesis is recommended to ensure animal welfare, clean sport and fair competition.

Urine is the most commonly used matrix in anti-doping control (Toutain, 2010b). However, in terms of control of legal therapeutic substances, urine is not as suitable as plasma. The urine concentration is often decided by the plasma concentration and plasma concentration is most often a better surrogate marker for drug concentration at the target site. In addition, for some drugs renal clearance is low and the main route of elimination is via the feces. However, drug concentrations are often higher in urine and are also affected by, for instance, urine volume (*e.g.* during dehydration) so the relation between concentrations in plasma and in urine might be imprecise. Consequently, there is a risk of detecting traces of legitimate medication in urine when plasma concentrations might be considered without relevant effect. Urine dexamethasone data displayed higher inter- and intra-individual variation than plasma dexamethasone data in this thesis. This variation most probably reflects the horses' states of hydration since urine volume varies both over time and between individuals. In this thesis, this variation was not corrected for, since the aim of sampling urine was to obtain data for the control of therapeutic substances. Inspection of the dexamethasone urine and plasma concentration plot obtained here (Fig. 2) suggests a correlation between plasma and urine concentrations in the population studied, with a urine/plasma ratio in the

terminal phase of approximately 10. Applying this ratio would suggest a urine SL of 10 pg·mL^{-1} based on a plasma SL of 1 pg·mL^{-1} and the model proposed by Toutain & Lassourd (2202). Unfortunately, there is no international SL published for dexamethasone in plasma. The proposed SL of 1 pg·mL^{-1} can be compared with the threshold of 5 pg·mL^{-1} used by the Association of Racing Commissioners International (ARCI) to estimate WT after administration of DSP (ARCI, 2016). In urine, the SL of 10 pg·mL^{-1} presented in this thesis can be compared with the published international SL of 200 pg·mL^{-1} (IFHA, 2017b)

6.3.2 Detection times and withdrawal times

After im administration of dexamethasone-21-isonictinate, dexamethasone was quantifiable in plasma (LLOQ 25 pg·mL^{-1}) for 13 days and in urine (LLOQ 150 pg·mL^{-1}) for 14 days. This is equivalent with the DT proposed by EHSLC, which is based on the SL used by EHSLC for dexamethasone (EHSLC, 2015). To allow the dexamethasone plasma concentration to drop below 5 pg·mL^{-1} in all horses, this time would be extended by 6 days using the terminal half-life for dexamethasone in plasma presented in this thesis (Table 3).

Paper I used six horses with high between-animal variation in elimination rate. The DT should therefore be doubled to produce a WT (Toutain, 2010a). It is important to mention that the figures presented in this thesis have not been approved by any regulatory organisations or any other organisation associated with racing or equestrian sports (*e.g.* EHSLC). A WT is commonly a recommendation chosen by the treating veterinarian after all circumstances are accounted for to minimise the risk of positive findings in doping-control on race day (EHSLC, 2012). In the Nordic countries, recommendations similar to WT are given by the racing authorities. In Paper II DSP was administered iv as a bolus + constant rate infusion regimen in doses different from the approved doses (Medical Products Agency, 2015). In paper III, DSP was administered ia. Only the 3 mg dose is consistent with an approved dose (Medical Products Agency, 2015). Therefore, the dexamethasone data presented cannot be used to determine DT. The EHSLC reports the DT 5 days after iv administration of DSP (EHSLC, 2015). There is no DT information available after ia administration of DSP.

6.3.3 Anti-doping control after intraarticular administration

The dexamethasone plasma concentrations were 200-fold to 10000-fold lower than the dexamethasone synovial fluid concentrations (Figure 6). At observations made longer after the time of injection the difference between plasma and synovial fluid concentrations were smaller compared with observations closer to the time of injection. The relatively large and variable range of

concentration difference indicates low success rate in extrapolating concentrations from synovial fluid to plasma and *vice versa*. Dexamethasone plasma concentrations also fell below the LLOQ of the analytical method before synovial fluid concentrations did. Detection of dexamethasone concentrations in plasma or urine after ia injection of DSP suggests that the horse was treated relatively recently. However, this thesis suggests that neither urine nor plasma is a good indicator to predict dexamethasone synovial fluid concentration. This was previously discussed by Toutain (2010b) and similar conclusions have been reached after ia injection of other glucocorticoids (Knych *et al*., 2013; Knych *et al*., 2014; Knych *et al*., 2016; Knych *et al*., 2017a). To ensure animal welfare and protect the integrity of the sport methods other than taking anti-doping control samples during competition could be used. A stand-down period after ia glucocorticoid injection combined with compulsory medication records is one alternative approach to the problem. This should be combined with out of competition testing that enables comparison with the medication record and the laboratory report.

6.3.4 Can control of therapeutic substances be improved?

One future complement to the use of drug concentration in anti-doping control is the use of biomarker quantification. A biomarker on the causal pathway between drug exposure and response has the potential to increase reliability in predicting the intensity of the drug response compared with a single-time point observation such as drug plasma or urine concentration. This requires further studies on biomarker baseline, drug concentration-biomarker response relationship and establishment of biomarker thresholds for negative tests. The use of biomarkers would also increase the possible time for detection in control of substances with short plasma/urine half-life and long duration of response. The use of an enlarged anti-doping control methodology would increase understanding as well as trust in control of therapeutic substances among both the public and practitioners in racing and equestrian sport, which improve animal welfare and protect the integrity of the sport.

7 Conclusions

The overall aim of this thesis was to study dexamethasone exposure and response in Standardbreds by means of quantitative methods. The work described in the thesis comprises studies on horses treated with dexamethasone intramuscularly, intravenously and intraarticularly. The dexamethasone concentration in plasma and synovial fluid was found to be related to the response. The dexamethasone urine concentration appeared to be related to the plasma concentration. The results obtained allow the following conclusions to be drawn on the dexamethasone concentration-response relationship.

- Dexamethasone concentration-time courses in plasma and synovial fluid was quantified after intramuscular, intravenous and intraarticular administration. The dexamethasone concentration-time course in urine was similar in shape to that in plasma but concentrations were higher at equivalent time points. The disposition in Standardbreds is consistent with Thoroughbreds.
- Dexamethasone is potent and efficacious. A dexamethasone concentration-response relationship was shown. The clinical endpoint lameness was suppressed at doses lower than approved doses.
- Cortisol baseline and cortisol response were quantified and the results were used to suggest improvement of a diagnostic test.
- IL-1β and lameness responses to dexamethasone treatment in an LPS-challenged joint were quantified. The relatively large difference in potency compared to cortisol response suggests that cortisol is not a suitable biomarker for the anti-inflammatory response to dexamethasone.
- A low screening limit for dexamethasone was indicated. Information on dexamethasone exposure was provided. An optional strategy in anti-doping control of therapeutic medication after intraarticular administration of dexamethasone was proposed. Plasma and urine were found not to be good indicators of synovial fluid dexamethasone concentration.

8 Future perspectives

New knowledge commonly produces new questions. This thesis provided information of dexamethasone that can be used in future research, dosing recommendations and anti-doping methodology. Quantitative information on other glucocorticoids used in horses would have the potential to increase animal welfare by means of optimised dosing regimens and improved anti-doping control.

Future identification of a biomarker that can be used to investigate other glucocorticoids with respect to their PD properties is warranted. The protein Annexin A1 might fit the criteria. In this thesis, unsuccessful attempts were made to quantify Annexin A1 in plasma. Future research is recommended to focus on validation of a quantitative method that instead uses the white blood cells where the protein is synthesised and stored.

Reliable PK and PD information provides a base for a dosage regimen. The screening of glucocorticoid substances by means of a biomarker strategy would provide data that indicate if the doses currently used are adequate or whether adjustments can be made.

The PK and PD properties of glucocorticoids used in sustained release formulations (*e.g.* triamcinolone acetate, betamethasone acetate and methyl-prednisolone acetate) should be characterised in the inflamed joint. The novel finding in this thesis that dexamethasone decrease lameness at doses lower than the approved doses (Medical Products Agency, 2015), combined with that glucocorticoids have potentially detrimental impact on cartilage turnover in a dose-dependent manner, indicate that lower doses would be safer and improve animal welfare.

Implementation of a biomarker strategy might be viable a complement to drug concentrations in anti-doping control in racing and equestrian sports.

9 Populärvetenskaplig sammanfattning

Dexametason är en substans inom en grupp anti-inflammatoriska läkemedel som i dagligt tal kallas för kortison. Kortison är vanligt för behandling av både tävlingshästar och sällskapshästar mot inflammation i leder, senor, muskler m.m. Läkemedlen kan ges till hästen på flera sätt, till exempel via munnen eller injiceras i blodet, i muskeln eller direkt i en led. Kortisonpreparat är effektiva läkemedel som gör stor nytta men tyvärr är de också förknippade med flera biverkningar, framförallt vid höga doser eller längre tids medicinering.

För att upprätthålla en god djurvälfärd är det viktigt att hästar får god vård när de är sjuka eller skadade. Det är också viktigt att hästar inte tävlar när de är påverkade av läkemedel, både av djurvälfärdsskäl samt för dopningsfri sport. Olika hästsportorganisationer världen över har olika regelverk som reglerar medicinering av djur inför tävling. I Sverige regleras det även i Jordbruksverkets föreskrifter. De senaste årtiondena har analysmetoderna på anti-dopningslaboratorierna blivit mer känsliga och läkemedel kan detekteras vid så pass låga koncentrationer i blod eller urin att läkemedlet inte längre kan anses ha någon effekt. Läkemedel kan till och med påvisas i prov från en obehandlad häst som placerats i en box där en behandlad häst tidigare stått om inte boxen inte gjorts rent ordentligt. För att hantera den problematiken har häst-sportens organisationer infört så kallade rapporteringsnivåer (*screening limits*, SL). En SL är en koncentration i blod eller urin vid vilken läkemedlet inte har någon effekt. Vetenskaplig dokumentation ligger till grund för vilken koncentration som bestäms. Om läkemedelskoncentrationen i ett prov ligger under en angiven SL rapporteras det som ett negativt prov. Ytterligare en åtgärd för att undvika dopningsutredningar vid regelrätt utförd medicinering är införande av detektionstider, *detection times* (DT) som är ett mått på hur lång tid det tar efter behandling med ett läkemedel till dess att koncentrationen är lägre än dess respektive SL. Utanför Skandinavien och Finland är det därför vanligt att den behandlande veterinären lägger till en säkerhetsmarginal till en DT för att

erhålla en *withdrawal time* (WT), en tid som bör förflyta mellan sista medicinering och start för att säkerställa att ett eventuellt dopningsprov blir negativt. I Skandinavien och Finland motsvaras WT närmast av de fastställda karenstiderna.

I arbetet inför den här avhandlingen har kortisonsubstansen dexametason givits till hästar i syfte att studera hur den tas upp, fördelas och försvinner från hästen. Koncentrationen dexametason har även relaterats till olika biomarkörer (ämnen som används som surrogat för att mäta läkemedelseffekten) och kliniska effekter för att kunna utvärdera behandlingsresultatet bland annat med så kallade kvantitativa metoder. Resultatet kan sedan användas i antidopningsarbetet, till exempel som underlag för att beräkna SL eller för att utvärdera de doser som används idag.

I studie I mättes dexametasonkoncentrationen i blod och urin efter en injektion i muskeln av ett långtidsverkande preparat. Läkemedlet är utformat för att bilda en depå i muskeln och långsamt distribueras ut i blodet för att få effekt under längre tid. Läkemedelskoncentrationerna i blodet följdes över tid och tiden det tar att minska koncentrationen i blodet med 50 % (halveringstiden) beräknades. Med hjälp av halveringstiden går det att beräkna hur lång tid det tar för koncentrationen i blodet att sjunka till en bestämd koncentration, till exempel en SL. Läkemedelskoncentrationen i blodet relaterades också till hämningen av det kroppsegna hormonet kortisol och koncentrationen som ger halva maximala effekten beräknades.

Vattenlösligt dexametason injicerades direkt i blodet i studie II och i inflammerad led i studie III, varpå prover togs och läkemedelskoncentrationen i blod och ledvätska fastställdes. Läkemedelskoncentrationen i plasma eller ledvätska minskar med hälften inom loppet av några timmar, varför upprepade injektioner krävs för att få effekt om längre tids behandling är nödvändig. Därefter bestämdes ett antal parametrar som anger hur läkemedel tas upp, fördelas och försvinner ur kroppen genom så kallad farmakokinetisk (PK) modellering. Med hjälp av resultatet från PK-modelleringen gick det även att relatera läkemedels-koncentrationen till effekten, med hjälp av så kallad farmakodynamisk (PD) modellering. Resultater styrkte också resultatet från studie I. Det har tidigare föreslagits att det inte går att relatera till exempel plasmakoncentrationen av kortison (glukokortikoider) till effekt, vilket den här avhandlingen således har motbevisat.

Det kroppsegna hormonet kortisol visades vara en känslig biomarkör för dexametason i blod. Att kortisolkoncentrationen i blodet sjunker efter behandling med kortison är väldokumenterat och används till exempel vid utredning och diagnostik av så kallad Cushings sjukdom. Användandet av

farmakologiska modeller möjliggjorde utvärdering av ett sådant testprotokoll med konkreta förslag på förbättringar som underlättar tolkningen av testsvaret.

De två sista studierna undersökte effekten av dexametason efter att en mild inflammation skapats i leden med hjälp av så kallade lipopolysackarider. Hästarna behandlades sedan med dexametason eller koksalt (placebo) direkt i leden. Om inflammationen behandlades med koksaltlösning blev hästarna halta i upp till 32 timmar. Efter injektion med dexametason direkt i leden lindrades eller släcktes hältan vid doser lägre än de som är godkända för ledinjektion i Sverige.

Resultaten i den här avhandlingen kan användas både som underlag för att beräkna DT och SL samt jämföras med (och i framtiden eventuellt revidera) etablerade behandlingsprotokoll för kortisontypen dexametason för att minska risken för biverkningar. För andra kortisontyper och för de som är långtidsverkande behövs mer information om hur de omsätts i leden samt hur mycket som behöver finnas i leden för att få en god effekt. Fram till dess att den kunskapen finns är det på sin plats att vara restriktiv i doseringen av de kortisonläkemedlen. Det finns sedan tidigare forskning som pekar på att kortison kan påverka ledbrosk negativt och högre doser kan potentiellt ge större negativa effekter. Ett behandlingsprotokoll med lägre doser skulle kunna leda till färre oönskade effekter vilket är önskvärt. Det är viktig information att ta reda på för att i framtiden eventuellt kunna revidera behandlingsprotokollen och säkra en trygg och effektiv behandling samt en pålitlig kontroll av tillåtna mediciner inom anti-dopningsarbetet, vilket är betydelsefullt för djurvälfärden.

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