

# **Drugs in Horses: Pharmacokinetics and Pharmacodynamics**

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**Poison is in everything, and no thing is without poison.  
The dosage makes it either a poison or a remedy.**

*Philippus Theophrastus Aureolus Bombastus von Hohenheim  
(1493-1541)*

*Also known as Paracelsus - the father of toxicology*



## Abstract

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In this thesis the fate and effect of some drugs have been examined in horses. Studies have also been performed to explore some factors which may affect the pharmacokinetics and the pharmacodynamics of drugs in horses.

Investigations on the drug metabolising enzyme cytochrome P450 3A (CYP3A) in the intestines of horses showed high gene expression and metabolic activity in the proximal parts of the intestines. The results indicate that CYP3A in the intestines of horse plays a major role in the first-pass metabolism of drugs which are substrates for CYP3A.

There is a need for an antihistamine for oral therapy of horses. The oral bioavailability of the antihistamine fexofenadine was found to be low, and this drug is therefore unsuitable for oral use in horses. In contrast oral administration of the antihistamine cetirizine resulted in a sufficient uptake. This drug was also found to have a potent antihistaminic effect in horses. Cetirizine may therefore be a suitable antihistamine in equine medicine.

The passage of antihistamines, such as fexofenadine and cetirizine, as well as several other xenobiotics, over cell-membranes in various tissues is partly regulated by transport proteins. Studies in this thesis showed that pre-treatment of horses with the antiparasitic agent ivermectin affects the oral bioavailability of fexofenadine and cetirizine. The effect of ivermectin is probably related to interference in the function of the transport proteins.

Acute adverse reactions may occur following treatments of horses with procaine benzylpenicillin or potassium or sodium benzylpenicillin. Analysis of adverse reactions reported in 59 horses indicates that allergy may underlie a few of the cases. However, most reactions may be due to toxic effects of procaine. Several mechanisms may contribute to the procaine toxicity. It was shown that the ability of plasma esterases to hydrolyze procaine to non-toxic metabolites was lower in reacting horses compared to non-reacting control horses. Low plasma esterase activity may increase the likelihood for procaine toxicity and constitute one risk factor.

*Keywords:* horse, CYP3A, intestine, pharmacokinetics, pharmacodynamics, antihistamine, cetirizine, fexofenadine, ivermectin, drug interactions, adverse reaction, penicillin, procaine, plasma esterase

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# Contents

## **Introduction, 11**

Background, 11

The fate of drugs in the body, 12

Anatomy and physiology of the equine intestines, liver and kidney, 14

Metabolising enzymes, 15

*CYP-enzymes, 16*

Membrane transport, 17

*P-gp, 17*

*OATP, 18*

Adverse drug reactions, 18

*Drug interactions, 19*

Tools for studying the fate and effect of drugs in horses, 20

*CYP3A studies, 20*

*Pharmacokinetic, pharmacodynamic and interaction studies, 21*

*Esterase activity studies, 22*

Examined drugs, 22

*Antihistamines, 22*

*Ivermectin, 25*

*Benzylpenicillin, 25*

*Procaine, 25*

## **Aims, 27**

## **Material and Methods, 28**

Horses, 28

Real-time RT-PCR, 28

Immunohistochemistry, 28

CYP3A enzyme assay, 29

Drug administration, 29

Blood sampling, 29

Recording of the pharmacodynamic effect, 29

Analysis of antihistamines, 30

Analysis of PABA, 30

Pharmacokinetic analyses, 30

Pharmacokinetic/pharmacodynamic link model, 32

Statistical analysis, 33

## **Results and Discussion, 34**

Occurrence and activity of CYP3A in the intestines, 34

Fate of the antihistamines and interactions with ivermectin, 34

Effects of the antihistamines and PK/PD modelling, 36

Adverse reactions to benzylpenicillin, 38

**Conclusions, 40**

**Future perspectives, 41**

**References, 42**

**Acknowledgements, 50**

**Populärvetenskaplig sammanfattning, 52**



# Appendix

## Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Tydén, E., Olsén, L., Tallkvist, J., Larsson, P., Tjälve, H. (2004) CYP3A in horse intestines. *Toxicology and Applied Pharmacology*. 201: 112-119.

II. Olsén, L., Ingvast-Larsson, C., Larsson, P., Broström, H., Bondesson, U., Sundqvist, M., Tjälve, H. (2006). Fexofenadine in horses: pharmacokinetics, pharmacodynamics and effect of ivermectin pre-treatment. *Journal of Veterinary Pharmacology and Therapeutics*. 29:129-135.

III. Olsén, L., Ingvast-Larsson, C., Broström, H., Bondesson, U., Tjälve, H., Larsson, P. (2007). Cetirizine in horses: pharmacokinetics and effect of ivermectin pre-treatment. *Journal of Veterinary Pharmacology and Therapeutics*. Accepted for publication.

IV. Olsén, L., Bondesson, U., Broström, H., Tjälve, H., Ingvast-Larsson, C. (2007). Cetirizine in horses: pharmacokinetics and pharmacodynamics following repeated oral administrations. *The Veterinary Journal*. Accepted for publication.

V. Olsén, L., Ingvast-Larsson, C., Broström, H., Larsson, P., Tjälve, H. (2007). Clinical signs and etiology of adverse reactions to procaine benzylpenicillin and sodium/potassium benzylpenicillin in horses. *Journal of Veterinary Pharmacology and Therapeutics*. Accepted for publication.

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## Abbreviations

ATP	adenosine triphosphate
AUC	area under the plasma concentration time curve
AUMC	area under the first moment curve
b.w.	body weight
cDNA	complementary DNA
Cl	clearance
$C_{max}$	the maximal plasma concentration
CYP	cytochrome P450
DFB	3-(3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-5 <i>H</i> -furan-2-one
DFH	3-hydroxy-5,5-dimethyl-4-(4-methylsulfonylphenyl)-5 <i>H</i> -furan-2-one
DNA	deoxyribonucleic acid
$E_{max}$	the maximal effect
EC <sub>50</sub>	the plasma concentration producing 50 % of the maximal effect
F	bioavailability
HPLC	high-performance liquid chromatography
IgE	immunoglobulin of type E
i.m.	intramuscular
i.v.	intravenous
$K_{e0}$	the rate constant for the elimination of drug from the effect compartment
LC-ESI-MS/MS	liquid chromatography electrospray ionisation tandem mass spectrometry
MDR1	multidrug resistance gene 1
mRNA	messenger RNA
MRT	mean residence time
NADP	nicotinamide adenine dinucleotide phosphate
NSAID	non-steroidal anti-inflammatory drugs
OATP	organic anion transport polypeptide
PABA	para-aminobenzoic acid
pH	negative logarithm of the hydrogen ion concentration of an aqueous solution
PD	pharmacodynamics
P-gp	permeability-glycoprotein
PK	pharmacokinetics
pK	negative logarithm in an equilibrium constant; pKa for acids
p.o.	oral
Real-time RT-PCR	real time reversed transcription polymerase chain reaction
RNA	ribonucleic acid
s.c.	subcutaneous
ss	steady state
$T_{max}$	time to obtain the maximal plasma concentration
$t_{1/2}$	half-life
$V_d$	volume of distribution

# Introduction

## Background

Knowledge about the factors, which determine the fate of drugs in the body is essential in order to get the right drug and dosage schedules and also to avoid toxic effects. Right doses mean that the horse will receive maximum benefit, in terms of optimal efficacy with minimal toxicity. Correct drug dosing will contribute a lot to the animal's welfare.

Horses, like other animals, have evolved complex systems that detoxify foreign chemicals, such as toxins present in poisonous plants. As concern the mechanisms by which the body handles foreign compounds, there is no difference between drugs and other xenobiotics. In order to exert their effects, drugs need to achieve adequate concentrations in the target tissue(s). Drug concentrations in the body is depending on the drug disposition, which in turn, is depending on the absorption from the site of administration, the distribution within the body, the metabolism and the excretion.

Oral administration is a convenient way to deliver a drug, but this route also has considerable challenges. Drug absorption from the gastrointestinal tract is a complex process affected by factors such as the rate of stomach emptying, the intestinal motility, the environment in the intestinal lumen, the structure of the intestinal mucosa and the presence and distribution of metabolising enzymes and drug transport proteins in the intestinal wall and liver. When the drug molecule reaches the bloodstream, the cardiovascular system provides a very fast long-distance bulk flow distribution arrangement. The way in which drugs cross the cell membranes differ markedly between different drugs due to their chemical and physical properties. This transfer is difficult to predict and is depending of several factors such as chirality, solubility, lipophilicity, pKa, hydrogen bonding capacity, molecular size and weight. The transport of drugs across cell-membranes may also be regulated by transport proteins. Such processes are especially important in the renal tubules, the biliary tract, and the gastrointestinal mucosa and at the blood-brain barrier.

Drug elimination is the irreversible loss of drug from the body and occurs by metabolism and excretion. The rate of the metabolism is one important factor that determines both the concentrations and durations of the drug in the body. A large number of enzymes are involved in the metabolism of drugs. The liver plays a major role in the first-pass metabolism of orally administered drugs and also regulates the systemic drug levels. High levels of metabolising enzymes are expressed also in several other tissues. Thus, metabolism in the intestinal epithelium may contribute to the first-pass metabolism of drugs. Drug metabolism in other extrahepatic tissues can lead to alteration of the efficacies of drugs by inactivation of active drugs or by activation of prodrugs but usually has limited influence of the overall elimination rate. Several compounds that cause toxicity are metabolically activated to reactive intermediates that bind to tissue

macromolecules locally in the tissues in which the metabolism takes place. Drugs may also interact on a chemical-physical, physiological, pharmacodynamical or pharmacokinetical basis, which in turn can lead to unwanted or no effects of the drug.

The excretion occurs mainly via bile and urine. In the liver drugs or drug-metabolites are excreted into bile and are then eliminated via faeces. Urinary drug-excretion is depending on glomerular filtration as well as active and passive secretion and absorption processes in the renal tubular epithelium.

### **The fate of drugs in the body**

Understanding the mechanisms of drug action and exposure-response relationships are critical for dose selection. For the determination of the optimal dosing regimen, including dose, dosing interval and duration of treatment a dose-titration study is often used either in animal models or in *in vitro* systems. To assess this different pharmacokinetic (PK) parameters are used as tools. The goal is to obtain appropriate efficacy in the treated animal and to avoid adverse effects.

Bioavailability (F) is a term that quantifies the proportion (often measured in %) of a drug, which is absorbed and available to produce systemic effects (Toutain & Bousquet-Mélou, 2004a). F is defined by the European Medicines Evaluation Agency (EMA, human guidelines) as follows: 'bioavailability means the rate and extent to which the active substance or active moiety is absorbed from a pharmaceutical form, and becomes available at the site of action' (Anon 2001). Since it often is difficult to collect samples from the target tissue blood or plasma/serum is often used as a substitute for the site of action.

There are several processes that may influence the oral bioavailability. Before the drug is absorbed across the apical cell membrane of the enterocytes the drug must be dissolved. The absorption can be limited by several processes in the lumen of the intestines and at the apical membrane of the enterocytes. Some conditions originate in physicochemical factors of the drug itself, such as the lipophilicity, solubility, size, conformation, ionisation and stability. Other processes are depending on pharmaceutical factors such as dosage, crystal form and dissolution rate of the drugs. Also physiological, biochemical and pathological factors in the walls of the intestines may affect the absorption. In addition co-administration of other drugs, ingestion of xenobiotics and presence of food in the intestinal lumen will have impact on the absorption of the drug. The absorbed drug is then facing the enzymatic capacity of the enterocytes. The fraction that escapes metabolism in the intestines may then undergo metabolism in the liver before reaching the systemic circulation. The events in the liver are dependent of the blood flow, the protein binding, and the influence of metabolising enzymes and presence of transport proteins in the hepatocytes.

Other important PK parameters which often are assessed to estimate the fate of the drug in the body and to estimate correct dosage regimen are clearance (Cl), the volumes of distribution ( $V_d$ ) and the half-life ( $t_{1/2}$ ) in the therapeutic interval. Cl

measures the overall ability of the body to eliminate a drug by scaling the drug elimination rate (amount per time) by the corresponding plasma concentration level. This is an important PK parameter being one determinant of the dosage rate.  $Cl$  multiplied by the therapeutic plasma concentration in steady-state (ss) conditions divided with the systemic bioavailability gives the correct dosing rate (Toutain & Bousquet-Mélou, 2004b). Terminal  $t_{1/2}$  is the time required to divide the plasma concentration by two after reaching pseudo-equilibrium (Fig. 1). The main clinical application of  $t_{1/2}$  is to select an appropriate length for the dosing intervals to maintain the therapeutic plasma concentration and avoid drug accumulation and delay in the time to reach ss conditions.  $T_{1/2}$  is a hybrid parameter depending on several physiological factors and is linked both to the total body  $Cl$  and  $V_d$  as well to the drug absorption rate in case of flip-flop phenomena, where the absorption rate constant is lower than the elimination rate constant (Toutain & Bousquet-Mélou, 2004c).  $V_d$  is the proportionality constant between total amount of drug in the body and plasma concentration. Plasma concentrations may be measured at different conditions e.g. at equilibrium or pseudo-equilibrium and therefore several  $V_d$  have been defined.  $V_{d,area}$  is the appropriate  $V_d$  to consider during the terminal phase of a pseudo-equilibrium, whereas  $V_{d,ss}$  is the appropriate  $V_d$  to consider under ss conditions. A drug with large  $V_d$  can be selected in drug development to obtain a long terminal  $t_{1/2}$  even for drugs having a relatively high  $Cl$  (Toutain & Bousquet-Mélou, 2004d).

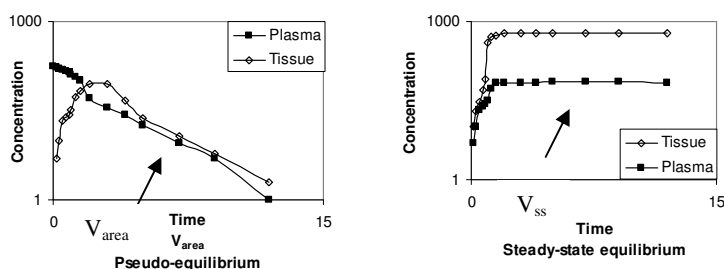


Figure 1. The difference between a pseudo-equilibrium state of distribution and an equilibrium condition.  $V_{area}$  is the appropriate  $V_d$  to consider during the terminal phase of a pseudo-equilibrium, whereas  $V_{ss}$  is the appropriate  $V_d$  to consider under steady-state conditions. Adopted from Toutain & Bousquet-Mélou 2004d.

Variability in therapeutic response and toxicity to drugs can be related to variation in PK and/or pharmacodynamics (PD). There may be considerable variations between species, and also between and within breeds. Other factors of impact are age, gender, and body weight. Physiological factors such as blood flow to various organs and body fat content may also affect drug distribution and elimination. For animals in which group dosing or pour-on products are used the administered dose is not precise, which may lead to variable clinical response. Also the owners' compliance with the prescribed dosage may play a role. Poor compliance may lead to inadequate responses and/or risk for toxicity. Anatomical and physiological differences in the intestines, liver and kidney may influence this variability.

## Anatomy and physiology of the equine intestines, liver and kidney

Being a herbivore the horse (*Equus caballus*) is highly specialised for a plant diet (Fig. 2). This has entailed a specialised dentition, since plant food must be thoroughly ground before the digestive enzymes can act upon it. The gastrointestinal tract of the horse is well adapted for its dual purpose of being important for the digestion and absorption of nutrients and also playing an important role as barrier against pathogens, toxins and xenobiotics. In a horse weighing about 500 kg the stomach has a volume varying from 8-15 litres and is remarkable small in size in relation to the animal and to the volumes consumed. The small intestine connects the stomach with the large intestine. It begins at the pylorus and terminates at the caecum. Its average length is about 22 metres, the average diameter is about 6-7 cm and the volume is about 40-50 litres. Its anterior part is the duodenum, which is followed by the mesenteric part, divided in jejunum and ileum. Both the duodenum and the ileum are about 1 m in length. The large intestine extends from the termination of the ileum to the anus and is about 7.5-8 m in length. It is divided in caecum, large colon, small colon and, rectum. The caecum is a large cul-de-sac between the small intestine and colon and has a capacity of 25-30 L. The ileocaecal sphincter controls the release of intestinal contents to the caecum and prevents colonic material to travel back into the small intestine. The large colon is 3-4 m in length and the diameter is 20-25 cm. Its capacity is more than double compared to caecum. The small colon begins at the termination of the great colon and is continued by the rectum. It is approximately 3.5 m in length its diameter is 7.5-10 cm. The epithelium of the small intestines is composed mostly of absorptive cells - the enterocytes- that occur in different stages of maturity. The epithelium of the large intestine has many similarities with that of the small intestine; the most obvious difference being that the epithelium in the colon lacks the folding and villi structure and that there is also a higher amount of mucus producing cells than in the small intestine. The intraluminal pH is crucial for the fate of many drugs. The pH is usually below 6 in the duodenum and then increases to about 7.5 in the jejunum (Merritt, 1999). It is important to know pKa values of drugs since this determines if they are present in an anionic, cationic or zwitterionic state. Also the transport velocity, or effective permeability, over cell membranes is important for the overall extent of drug absorption over the intestinal wall (Lennernäs, 1998; Davis *et al.*, 2006).

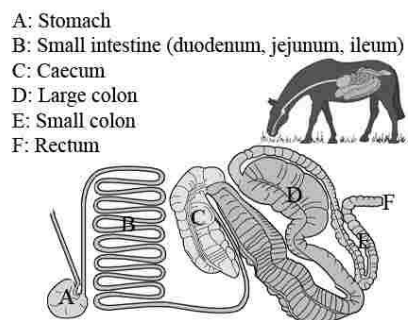


Figure 2. Schematic view of the abdominal tract of the horse.

The liver, which is the major metabolising organ in the body, has a dual blood supply, comprising the hepatic artery, which oxygenates the organ from the aorta, and the portal vein, which carries blood from the intestine. The blood vessels divide into smaller branches, which end in sinusoids. The sinusoids run between the cords of hepatocytes and give rise to a central vein. The functional units of the liver are the hexagonal lobuli termed acini. The blood in the hepatic sinusoids and the bile in the bile-canaliculi flow in opposite directions to each other. The bile-canaliculi are gathered to bile ducts, which are drained into the common bile duct. Horses have no gallbladder but a duct system which is highly developed, with a wide calibre of the bile ducts compensating for this. The bile is secreted in the duodenum. Compounds can be secreted into the intestine via the bile and then be reabsorbed (entero-hepatic cycling).

The kidneys, which remove xenobiotics and metabolic waste products from the blood, weigh about 700 g each. The left one has a conventional form but the right one is shaped more like a heart or spade of a playing card. The kidneys consist of an outer cortex and an inner medulla. The functional units called pyramids have nephrons and collecting ducts which empty the urine into the renal pelvis. In horses the kidneys are of a modified unipyramidal type, which implies that the pyramids are completely fused (the boundaries are revealed only by the arrangement of the interlobar arteries). In foals there is a clearer indication of lobation. The horse kidney contains 1-2 million urine-producing units (nephrons). Each nephron consists of a capillary network, glomerulus, and a tubular system lined with a continuous layer of epithelial cells and a basal lamina. The glomerular capillaries are surrounded by Bowman's capsule and the tubule distal to the capsule is divided in the proximal tubule, loop of Henle, distal tubule and collecting duct. The basic renal processes, i.e. glomerular filtration and tubular reabsorption and secretion, are similar to other mammals.

## **Metabolising enzymes**

The biotransformation of drugs and other xenobiotics can be divided in two phases. Phase I, is the initial stage, usually catalysed by cytochrome P450 (CYP) enzymes, and phase II, the conjugation stage catalysed by a second class of enzymes. In phase I a functional group is introduced or uncovered in the molecule through oxidation, reduction or hydrolysis (Timbrell, 2000; Guengerich, 2001; Nebbia, 2001; Isin & Guengerich, 2007). The altered molecule may then be conjugated to endogenous molecules in the phase II-reaction. The resulting conjugates are water-soluble and hence easily excreted via urine or bile. However, biotransformation of drugs and other foreign compounds may sometime lead to formation of reactive intermediates, which can react with proteins and DNA and thereby cause harmful effects (Klassen, 2001). The liver plays a major role in drug metabolism because of its large blood supply and its extensive biotransformation capacity. In addition, drug-metabolising enzymes are present in extrahepatic tissues, such as the kidney, the nasal olfactory mucosa and the mucosal linings of the respiratory pathways and the gastrointestinal tract. The enzymes in the intestinal mucosa are probably important contributors to first pass metabolism at the intestinal absorption of orally administered drugs (Lin & Lu, 2001; Isin & Guengerich, 2007).

### *CYP enzymes*

The cytochrome P450-enzymes (CYP-enzymes) are among the most important enzymes involved in the metabolism of drugs and other xenobiotics through the initial metabolism. CYP-enzymes are a group of heme-containing membrane-bound proteins present in the endoplasmic reticulum of the cells (Lin & Lu, 2001). To be enzymically active they require electrons. The first electron comes from NADPH via the accessory flavoprotein NADPH P450 reductase, a second electron may come from NADH P450 reductase or, in some cases, from cytochrome b<sub>5</sub> (Sheweita, 2000; Guengerich, 2001; Vleet *et al.*, 2002). CYP constitutes a multigene family, which consist of 27 gene families. At least 17 of these have been identified in mammals. The gene families are named with Arabic numbers (Voice *et al.*, 1999). They are divided into subfamilies and further into single distinct isoenzymes, such as CYP1A1 and CYP3A4. The subfamilies are designated by capital letters and the individual genes within the subfamily by Arabic numerals (Voice *et al.*, 1999; Guengerich, 2001). The mammalian CYP families can be functionally subdivided into two major classes: those involved the biosynthesis of endogenous molecules such as steroids, fatty acids and bile acid, and those involved in metabolism of xenobiotics (Lin & Lu, 2001). The three main CYP gene families, responsible for most of the xenobiotic biotransformation are CYP1, CYP2 and CYP3 (Timbrell, 2000). In CYP1 and CYP3 there is only one subfamily (A), whereas in CYP2 there are five subfamilies (A-E) (Voice *et al.*, 1999). CYP3A4 is the predominant isoform of CYP3A. This isoenzyme is involved in the biotransformation of many potentially harmful chemicals and commonly used drugs. Thus, CYP3A4 metabolises an array of structurally different xenobiotics (Klaassen, 2001). The levels of CYP3A4 may vary and the catalytic activity of this enzyme is important for the bioavailability of many drugs (Schwenk, 1988; Guengerich, 2001). Induction or inhibition of CYP3A4 appears to explain several well-known drug interactions (Guengerich, 1999). In cases where the biotransformation leads to metabolic activation, the reactive intermediates are unstable and therefore unlikely to be transported from one tissue to another or even from one cell to another. Thus, tissue-specific toxicity is usually related to local bioactivation in the sensitive tissues.

CYP enzymes have been extensively studied in humans and laboratory animals, whereas relatively little is known about these enzymes in domestic animals. In particular there are few reports on the presence and catalytic activity of these enzymes in extrahepatic tissues of domestic animals. There are only a few reports in the literature dealing with equine hepatic or extrahepatic CYP-enzymes (Nebbia, 2003, Larsson *et al.*, 2003). Since many toxic substances have their targets in the extrahepatic tissues studies on tissue-specific metabolism have a significant impact on the understanding of tissue-specific toxic effects. The extrahepatic tissues are usually morphologically heterogenous and the metabolising capacity may be confined to specific cells in the tissues. This can be exemplified by the localisation of CYP3A4 in the horse nasal olfactory mucosa which has been shown to be confined to the sustentacular cells in the surface epithelium and to cells of the Bowman's glands in the submucosa (Larsson *et al.*, 2003). It is known that there



sometimes are large species difference in biotransformation of drugs and other xenobiotics. There is a need of further studies aiming to characterise the expression, activity and localisation of the CYP enzymes in domestic animals, including horses.

## **Membrane transport**

Drugs can pass cellular membranes by passive transcellular diffusion, paracellular transport, endocytosis or carrier-mediated transport. In the intestinal mucosa passive diffusion is the most common route by which drugs passes the enterocytes. This process is driven by the concentration gradient, which implies that the compound moves towards regions of lower concentration (or by an electrochemical gradient). The paracellular transport is mainly considered to be a passive process and is probably of minor importance for drugs since it is mainly small compounds that are transported this way. Endocytosis is a specialised form of transport where particles are internalised by invagination of the apical cell-membrane into intracellular vesicles. There are several carrier-mediated transport mechanisms. When the compounds are transported across the membrane in the direction of the concentration gradient, although the transport has a saturable behaviour, it is called facilitated diffusion. Transporters that utilise ATP to maintain a gradient across the membrane are called primary active transporters. Secondary active transporters use energy from ion gradients (often Na<sup>+</sup>, Ca<sup>+</sup> and H<sup>+</sup> gradients) generated by primary active transporters. The phase II conjugates often have poor membrane permeability. Hence carrier-mediated transport is necessary for biliary excretion and secretion from hepatocytes into sinusoidal blood, and in addition for the excretion of drug metabolites from tubular cells of the kidney into the urine.

### *P-gp*

It has been shown that many drugs are effluxed by P-glycoprotein (P-gp). P-gp is a membrane protein that belongs to the ATP-binding cassette super family. These transporters are designated ABC-transporters. The main function of these transporters is the active efflux of various structurally unrelated exogenous compounds (coupled to ATP hydrolysis). The gene encoding for P-gp is termed multidrug resistance gene 1 (MDR1). The MDR transporter family may be overexpressed in cancers in response to chemotherapy and severely restrict antitumour drug efficacy. P-gp exerts a protective function against xenobiotic toxicity but, by limiting drug bioavailability, it may also reduce drug efficacy. It has been shown that many drugs that are metabolised by CYP3A are also effluxed by P-gp. P-gp is also important for the renal elimination of many drugs. In addition, due to its broad substrate specificity, P-gp may play a clinically relevant role in drug-drug (or drug-food) interactions (Loo & Clarke, 2005; Ambudkar, Kim & Sauna, 2006). Recent studies at our department have shown gene and protein expression of P-gp in enterocytes, hepatocytes and renal proximal tubular cells in horse (Tydén *et al.*, 2006).

### *OATP*

The organic anion transport polypeptides (OATP) are involved in the transport of a wide range of anionic, cationic and zwitterionic compounds mainly with a high molecular weight (Hagenbuch & Meier, 2003). It has been suggested that OATPs have the capacity of bidirectional transport and that they may be driven by an anion exchange or to coupling of the cellular uptake of organic compounds (Shi *et al.*, 1995; Satlin, Amin & Wolkoff, 1997; Li, Meijer & Ballatori 2000). It has been suggested that inhibition of OATP1A2 and/or OATP2B1 in the intestine by some fruit juices may explain the lowered systemic exposure of the antihistamine fexofenadine in man (Dresser *et al.*, 2002; Dresser, Kim & Bailey, 2005; Satoh *et al.*, 2005). There are as yet no reports in the literature on the occurrence of OATP in horse tissues.

### **Adverse drug reactions**

In horses, as in other species, adverse reactions can occur following treatments with various drugs. Adverse reactions to drugs can be of several different kinds, both caused by immune and non-immune mechanisms. Many of the adverse drug reactions are caused by predictable, non-immunologic mechanisms. This includes known side-effects related to treatments of certain drugs. Idiosyncratic or intolerance reactions are often unpredictable, non-immune drug reactions leading to overdose effects at therapeutic doses. Idiosyncratic reactions are defined as genetic predispositions for some drugs that occur only in a (often) small percent of the population. Drug intolerance is defined as a lower threshold to the normal pharmacological action of a drug. There are also unpredictable effects that may or may not be immune mediated. The immune mediated effects can involve different types of hypersensitivity: Type I reactions (IgE-mediated); Type II reactions (cytotoxic); Type III reactions (immune complex); and Type IV reactions (delayed, cell-mediated). The most common drug-related hypersensitivity is Type I allergic reaction. This condition includes cross-binding of antigenic drug- or drug-metabolite-molecules to specific IgE-antibodies attached to mast cells. This results in release of histamine and other immunological mediators, which cause various manifestations of hypersensitivity, such as urticaria, angioedema and anaphylaxis. Important drug-related risk factors for drug hypersensitivity are related to the chemical properties and molecular weight of the drug. Drugs of high molecular weight and high structural complexity are more likely to be immunogenic than drugs with small molecular weight. Another factor of importance for hypersensitivity drug reactions is the route of drug administration; subcutaneous (s.c.), i.m. and i.v. administrations are more likely to cause hypersensitivity reactions compared to oral administration. This could be explained by the effectiveness of antigen presentation in the skin, the adjuvant effects of depot drug preparations after i.m. administrations, and the high concentrations of circulating drug antigen rapidly achieved at i.v. therapy.

In horses, there are several reports of different kinds of adverse reactions to different kind of drugs (Tjälve, 1997; Woodward 2005). Vaccines may cause

injection site reactions, with stiffness, fever and depression (Tjälve, 1997). For anaesthetics, sedatives and analgesics, negative effects can be urticaria, local reaction on the site of injection or excitation (Tjälve, 1997). Non-steroidal anti-inflammatory drugs (NSAID), such as phenylbutazone, can cause gastric and intestinal ulceration, sometimes leading to peritonitis and death (Brumbaugh, 2001).

Adverse effects to antimicrobial drugs are reported relatively frequently in horses (Gray *et al.*, 2003; Dyer *et al.*, 2005). Thus, both oral and i.v. treatments with trimethoprim-sulfonamides have been associated with collapse and death (Tjälve, 1997). Fatal diarrhoea has been reported in association with antibiotics especially erythromycin but also trimethoprim and sulfonamides (Wilson *et al.*, 1996; Båverud *et al.*, 1998; Stratton-Phelps, Wilson & Gardner 2000).

Penicillin has a wide safety margin and is usually well tolerated. Yet adverse reactions other than gastrointestinal disturbances are observed (Marshall, 1980; Allpress & Heathcote, 1986; Nielsen *et al.*, 1988; Tjälve, 1997). Thus, it is well known that acute adverse reactions may occur following i.v. or i.m. injections of benzylpenicillin in horse. Penicillin, or its metabolites, have an affinity for proteins and may form hapten conjugates, which participate in immunological reactions. However, hypersensitivity reactions to penicillin have been reported to be relatively rare in animals, although the number and occurrence are not known in detail (Wilcke, 1986; Davis, 1987). Benzylpenicillin is used as highly water-soluble sodium or potassium salts or as a procaine salt with low water-solubility. The sodium and potassium salts are usually injected i.v., whereas the procaine salt is used as an i.m. depot preparation. The latter must be dissolved into free procaine and benzylpenicillin before absorption to the blood, implying that therapeutic benzylpenicillin-concentrations will be maintained for at least 24 hours (Uboh *et al.*, 2000). As regards procaine benzylpenicillin release of a high concentration of free procaine in the circulation may lead to systemic toxicity. The risk factors for the adverse reactions to benzylpenicillin in horses are discussed in this thesis.

### *Drug interactions*

When two or more drugs are administered over an overlapping time period, there is a possibility of drug interactions. Drug interactions are important causes of drug related toxicity (Kaufman & Shapiro, 2000). A drug interaction may be defined as occurring when the pharmacological or clinical response to a combination of drugs is different from that anticipated from the known effects of the single drugs. The net effect may be synergism, antagonism, alteration of the effect of one or more of the drugs, or idiosyncratic effects. A number of drugs may interact simultaneously at different sites, so it can be difficult to attribute an interaction to a single mechanism (Ito, Brown & Houston, 2004). There are several plausible mechanisms underlying drug interactions. A drug with a high affinity for a receptor may displace another drug with lower affinity from the receptor. Also alteration in the levels of transport proteins can affect the plasma concentrations of drugs. Inhibition or induction of the enzymes involved in the metabolism of drugs may occur. Inhibited drug metabolism may result in undesirable elevations in plasma

concentrations of drugs or metabolites, which in turn can lead to serious adverse effects (Kolars *et al.*, 1993). Enzyme induction may increase the drug's metabolism and elimination rate and deteriorate its pharmacological effect as a result of decreases in plasma concentrations. It has been proposed that P-gp and CYP3A are functionally linked and may act in concert to limit the passage of drugs across the enterocytes (Benet & Cummins, 2001). Herbs or herbal remedies and fruit juices may also affect the levels and activities of P-gp and CYP3A. In this thesis the interaction of ivermectin, which is a known substrate and inhibitor of P-gp, on the pharmacokinetics of the two antihistamines fexofenadine and cetirizine has been examined.

### Tools for studying the fate and effect of drugs in horses

There are different ways to study the fate of drugs in the horse. In this thesis both *in vitro* and *in vivo* methods were used.

#### *CYP3A studies*

The mRNA expression, immunohistochemical localisation and catalytic activity of CYP3A in the intestines of horses were examined using an array of different *in vitro* techniques. For detection of CYP3A-mRNA in the intestines a real-time reversed transcription polymerase chain reaction (RT-PCR) was used. Immunohistochemistry was used to trace the cellular localisation of CYP3A. The activity of CYP3A in microsomal fractions was determined with the CYP3A selective probe DFB (3-(3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-5H-furan-2-one). DFB is metabolised to the fluorescent metabolite DFH (3-hydroxy-5,5-dimethyl-4-(4-methylsulfonylphenyl)-5H-furan-2-one; Fig. 3).

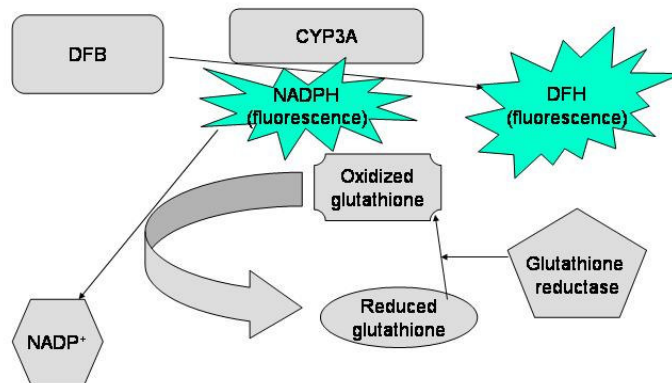


Figure 3. The formation to the fluorescent metabolite 3-hydroxy-5,5-dimethyl-4-(4-methylsulfonylphenyl)-5H-furan-2-one (DFH) from 3-(3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-5H-furan-2-one (DFB) by CYP3A-enzymes.

*Pharmacokinetic, pharmacodynamic and interaction studies*

Horses were used in clinical *in vivo* trials to study two different antihistamines by blood sampling and PK analyses. The PD, measured as the antihistaminic effect was assessed by intradermal histamine injections and then measurements of the reactions. Interaction studies were performed by examination of effect on the PK of the antihistamines after pre-treatment with ivermectin, which like the antihistamines, is a substrate for P-gp.

PK deals with the time course of drugs in the body. Specifically it is the study of drug absorption, distribution and elimination. Parameters describing this are calculated by mathematical models and include F, area under the plasma concentration time curve (AUC), Cl,  $V_d$ , and  $t_{1/2}$ . There are several models available, such as compartmental or non-compartmental models, and there are guidelines for best fit using model selection, weighing schemes, residual analyses and other diagnostic tools.

PD may be defined as a study of actions of drugs on the body or on microorganisms or parasites within or on the body. The PD of any given drug can be studied at many different biophase levels from sub-molecular, molecular, cellular, tissue/organ system to whole body (i.e. at an organism level). It is essential that a relevant endpoint has been chosen to measure the drug action. The PD model relates the biophase concentration to the effect (Holford & Sheiner, 1981; Lees, Cunningham & Elliott, 2004).

PKs and PDs attempt to relate the interaction of a drug to a biological environment. There are models that aim at linking PK and PD. Generally PK/PD modelling requires three separate models: one model for PK data, one for PD data and a link model describing the passage of drugs from plasma to the biophase (Toutain *et al.* 1994; Toutain & Lees, 2004). Often the effect lags some time behind plasma concentration. Hence, concentration cannot be incorporated directly into a PD model. This can be illustrated by plotting the effect (Y-axis) versus the plasma concentration (X-axis) and follow the data points in chronological order; this will result in a hysteresis loop, which is a Greek word meaning coming late (Fig 4). The link model is used to deal with this problem.

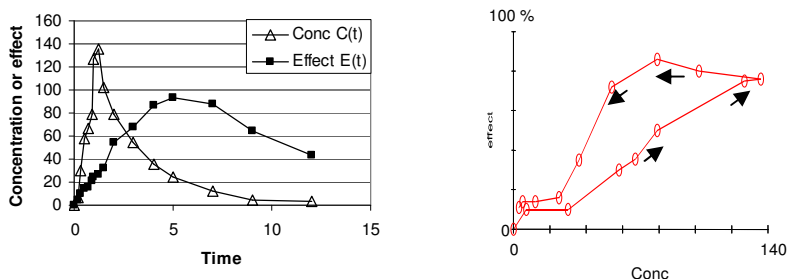


Figure 4. Plasma concentration-time and effect-time relationships are not in phase (left). The peak concentration occurs earlier than the peak effect. An arithmetic plot of effect versus concentration reveals a hysteresis loop (right).

#### *Esterase activity studies*

An *in vitro* experiment was used to assess the activity of plasma esterases in horses reacting adversely to procaine benzylpenicillin and in control horses. Following entry into the systemic circulation procaine is hydrolyzed by plasma esterases to the non-toxic metabolites *para*-aminobenzoic acid (PABA) and diethylaminoethanol (Fig. 5; Tobin *et al.*, 1976). To examine plasma esterase activity samples of horse plasma were incubated with procaine in a water-bath and the formation of PABA was then analysed by high-performance liquid chromatography (HPLC).

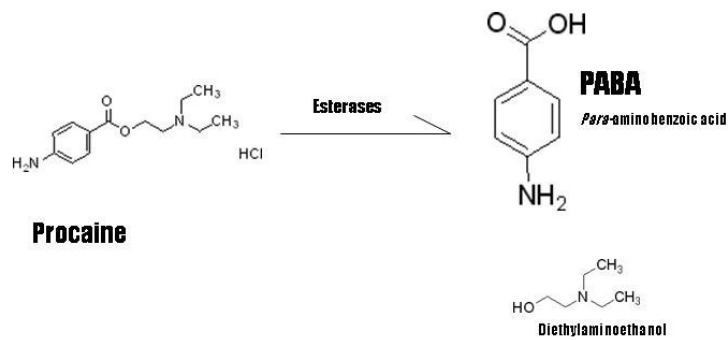


Figure 5. Hydrolysis of procaine to *para*-aminobenzoic acid (PABA) and diethylaminoethanol by plasma esterases.

## **Examined drugs**

### *Antihistamines*

There is a need for an antihistamine (histamine H<sub>1</sub>-receptor antagonist) with good bioavailability for oral therapy of allergic disorders in horse. In Sweden no such drug is approved for use in animals. However, antihistamines are tentatively used in veterinary medicine to treat hypersensitivity conditions related to effects of histamine. In hypersensitivity reactions, allergens cross-bind IgE on the surface of mast cells and basophils, thereby causing degranulation. Histamine is one of the inflammatory mediators released together with proteolytic enzymes, prostaglandins, leukotrienes and proinflammatory cytokines. The free histamine contributes to the different clinical signs of allergy, such as oedema, urticaria, eczema, discharges from mucous membranes and eyes and circulatory shock

syndromes (Adams, 1995). Antihistamines act as competitive antagonists for the histamine receptors in the tissues. In equine therapy histamine H<sub>1</sub>-antagonists such as tripeleminamine, promethazine and chlorpheniramine have been used in the treatment of hypersensitivity to biting insects of the *Culicoides spp.* (Morrow, Quinn & Baker, 1986; Rosenkrantz, 1995; Foster, McKelvie & Cunningham, 1998). Histamine H<sub>1</sub>-antagonists may also be used in other conditions in which histamine can be a critical mediator, such as allergic reactions to venoms and other antigens and drug induced anaphylactic shock (Adams, 1995). The antihistamines examined in this thesis are fexofenadine and cetirizine.

#### Fexofenadine

Fexofenadine (Fig. 6), a metabolite of terfenadine, is a selective non-sedating histamine H<sub>1</sub>-receptor antagonist that in man is clinically active in the treatment of seasonal allergic rhinitis and chronic idiopathic urticaria (Simpson & Jarvis, 2000). Fexofenadine has very little or no anti-cholinergic activity (Orzechowski, Currie & Valancius, 2005; Liu & Farley, 2005). In man fexofenadine is regarded as being safe, even at high plasma concentrations (Russell, Stoltz & Weir, 1998; Simpson & Jarvis 2000). Fexofenadine is metabolised only to a minor degree in man and rat (Lippert *et al.*, 1995; Kamath *et al.*, 2005). Fexofenadine is a zwitterion, with one carboxyl and one piperidine group, having pK<sub>a</sub> values of 4.25 and 9.53 respectively (Yasui-Furukori *et al.*, 2005).

#### Cetirizine

Cetirizine (Fig. 7), a metabolite of hydroxyzine, is a non-sedative second generation H<sub>1</sub> antihistamine widely prescribed and with demonstrated efficacy in the treatment of urticaria and pruritus in humans (Simons, 2001; Zuberbier & Henz, 1999). In man cetirizine has pharmacodynamically and pharmacokinetically favourable characteristics such as rapid onset of action and a low degree of metabolism. It is well tolerated, safe and has been shown not to alter memory, attention, alertness or performance in humans (Pagliara *et al.*, 1998; Benedetti *et al.*, 2001, Curran, Scott & Perry, 2004, Theunissen *et al.*, 2004; Theunissen, Vermeeren & Ramaekers, 2006). Cetirizine also displays high affinity and selectivity for cloned human H<sub>1</sub>-histamine receptors (Gillard *et al.*, 2002). At a pH between 3.5 and 7.5 cetirizine exists nearly exclusively as a zwitterion, and also appears to form partial intramolecular charge neutralisation in folded conformers of lower polarity (Pagliara *et al.*, 1998).

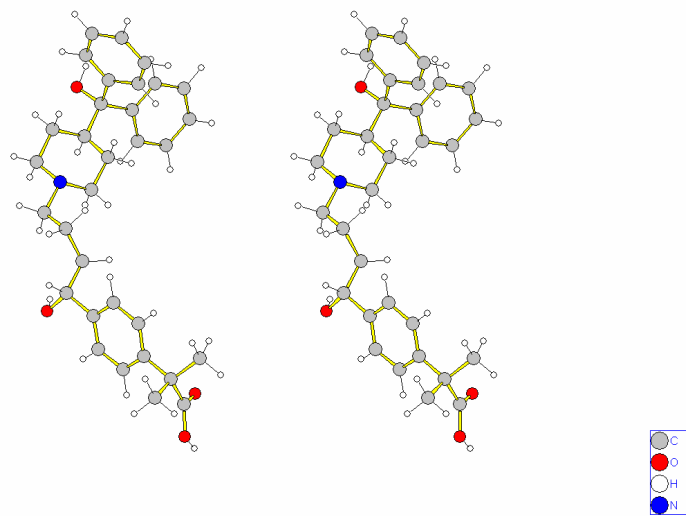


Figure 6. Stereo view of fexofenadine.

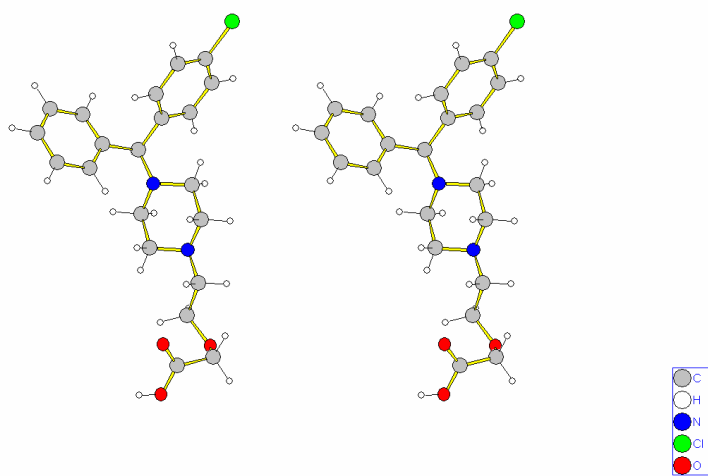


Figure 7. Stereo view of cetirizine.



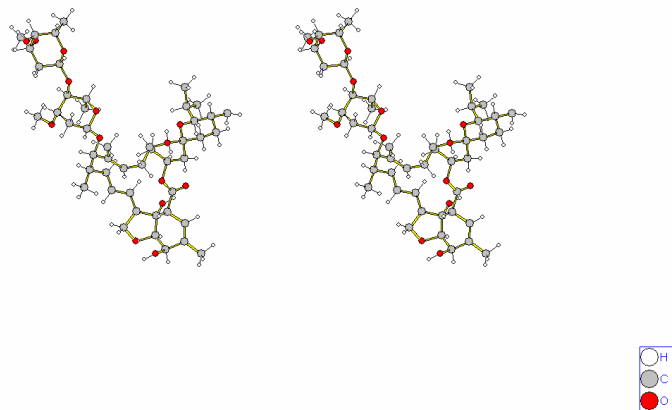


Figure 8. Proposed stereo view of ivermectin.

### *Ivermectin*

Ivermectin (Fig. 8) is a macrocyclic lactone. This constitutes a large family of structurally related compounds widely used for the treatment of both internal and external parasites (McKellar & Benchaoui, 1996). Ivermectin is a well-known substrate of P-gp (Schinkel *et al.*, 1994) and has also been shown to be a P-gp inhibitor (Didier & Loor, 1996). Ivermectin is used in this thesis for interaction studies with fexofenadine and cetirizine.

### *Benzylpenicillin*

Benzylpenicillin (Fig. 9) is the most frequently used penicillin-derivative in equine therapy. It is used as highly water-soluble sodium or potassium salts or as a procaine salt with low water-solubility. The sodium and potassium salts are usually injected i.v, whereas the procaine salt is used as an i.m. depot preparation. The latter must be dissolved into free procaine and benzylpenicillin before absorption to the blood, implying that therapeutic benzylpenicillin-concentrations will be maintained for at least 24 hours (Uboh *et al.*, 2000). Penicillin, or its metabolites, have an affinity for proteins and may form hapten conjugates, which participate in immunological reactions. (Downham *et al.*, 1978). In this thesis clinical signs and etiology of adverse reactions to procaine benzylpenicillin and sodium/potassium benzylpenicillin in horses are described.

### *Procaine*

Procaine (Fig. 9) forms a salt together with benzylpenicillin. The procaine delays the absorption of benzylpenicillin from the i.m. injection site. In addition it acts as a local anaesthetic, decreasing the discomfort of injecting a large volume of

benzylpenicillin into the muscle (Uboh *et al.* 2000). Inadvertent intravascular administration of the procaine benzylpenicillin may result in a rapid dissociation of the salt into procaine and benzylpenicillin when diluted in the large blood volume. Adverse reactions may then be induced due to an effect of free procaine on the CNS. Following entry into the systemic circulation procaine is hydrolyzed by plasma esterases to non-toxic metabolites (Tobin *et al.*, 1976). Procaine toxicity may occur when the amount of free procaine in the blood exceeds the hydrolysing capacity of the plasma esterases. In this thesis procaine was used in an *in vitro* experiment to assess if the horses reacting acutely to procaine benzylpenicillin have lower plasma esterase activity compared to non-reacting control horses.

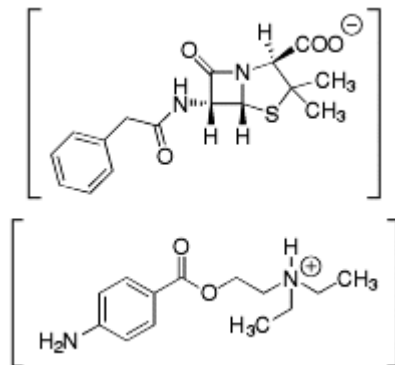


Figure 9. The structure of the procaine benzylpenicillin salt. The benzylpenicillin is shown above and the procaine below. Procaine benzylpenicillin has low water solubility. If procaine is exchanged by sodium ( $\text{Na}^+$ ) or potassium ( $\text{K}^+$ ) the more water-soluble Na/K benzylpenicillin salts are formed.

## Aims

The general aim of the present thesis was to increase the basic knowledge of some factors determining the fate and effect of some drugs in the horse.

The specific aims of this thesis were:

- to elucidate the regional distribution of gene expression and catalytic activity and the cellular localisation of CYP3A in the intestines in horses.
- to study the pharmacokinetics and the antihistaminic effect of the two antihistamines fexofenadine and cetirizine in horses.
- to examine if pre-treatment with ivermectin would affect the pharmacokinetics of fexofenadine and cetirizine in horses.
- to increase the knowledge of the etiology of the adverse reactions to benzylpenicillin in horses.

## Material and Methods

### Horses

In paper I tissues from seven Standard bred trotters, 3-9 years old, were collected from a local abattoir. In paper II five mares, 6-12 years old, were used. Three of the horses were Standard bred trotters, one was a Swedish warm blood riding horse and one was a New Forest. In paper III seven healthy Standard bred trotters (five mares and two geldings), 5-21 years old, were used. In paper IV six Standard bred trotters (mares), 5-21 years old, were used. In paper V information on adverse reactions to benzylpenicillin in 59 horses of different breeds were collected. The horses ranged in age from 8 months to 29 years and comprised 34 mares and 25 geldings. Within this group blood samples were collected from 31 horses (16 mares and 15 geldings aged 2-24 years). Seventeen horses of different breeds (8 mares and 9 geldings aged 2-20 years) were used as controls. The control horses had previously been treated with procaine benzylpenicillin without any adverse reactions. All study protocols were approved by the Animal Ethics Committee, Uppsala, Sweden.

### Real-time RT-PCR

In paper I, total RNA from intestinal mucosa and liver was prepared and the purity and the approximal concentration were determined spectrophotometrically. The samples were then run on an ethidium bromide agarose gel to check the integrity of the RNA. The primers were designed based on pig, rat and human CYP3A4 cDNA sequences. A gene-region conserved between the species was found. The pig cDNA was chosen as template. The sequences of the primers were 5'-CAG CCT GGT GCT CCT CTA TC-3'(forward) and 5'-TCA ACA CCC TTA CGG TAG CC-3'(reverse). The primers were assessed for functionality and specificity and then quantified by PCR reactions with an iCycler. At the time for this study the gene-sequence for equine CYP3A4 was unknown so we could not exclude that the primer-pair may generate PCR-products for other CYP3A isoenzymes. The RNA samples were pretreated with DNase (Huang *et al.*, 1996) and the exact RNA concentrations were measured in a microplate reader. The real-time RT-PCR reaction was carried out using Rotor-Gene 3000. Quantification was done using Rotor-Gene software and a melt curve analysis was done to check the specificity of the obtained PCR-product.

### Immunohistochemistry

In paper I, immunohistochemistry was used to trace the cellular localisation of CYP3A in the intestinal mucosa in various parts of the intestines and in the liver. Paraformaldehyde-fixed tissue pieces were dehydrated and embedded in paraffin. Five µm thick tissue sections were deparaffinised, hydrated and rinsed and then incubated with primary antibodies (rabbit anti-human) against CYP3A4. Secondary antibodies (goat anti-rabbit) were then applied to the sections, which thereafter were stained for visualisation of the CYP3A immunoreactivity.

### **CYP3A enzyme assay**

In paper I microsomes were prepared by homogenisation of the tissues from the intestinal mucosa and the liver followed by centrifugation twice at 10000 x g for 25 min. The supernatants were then centrifuged at 105000 x g for 1 hour. The protein contents of the microsomes were determined in a microplate reader (Smith *et al.*, 1985). The activity of CYP3A in the microsomal fractions was determined with a 96-well plate assay using the probe DFB. DFB is metabolised to the fluorescent metabolite DFH mainly by CYP3A4 and to a low extent also by CYP3A5 (Chauret *et al.*, 1999). An inhibition assay was also conducted using the CYP3A inhibitor ketokonazole.

### **Drug administration**

In papers II and III the i.v. administrations of antihistamines were given via a jugular vein catheter; the oral administrations were given via a nasogastric tube. Ivermectin, in a paste formulation for equine use, was given into the mouth. In paper IV the oral administrations were given mixed with sugar beet.

### **Blood sampling**

In papers II and III the blood samples were collected via a jugular vein catheter into test tubes with heparin as anticoagulant. In paper IV blood samples were collected via a jugular vein catheter after the last administration and at other time points via direct vein puncture. In paper V blood samples were taken from a jugular vein and collected into heparin vacutainer tubes and placed in an ice bucket. All samples were centrifuged at 1500 g at +4°C for 15 min to collect plasma. In paper II, III and IV plasma was stored at -20°C and in paper V at -70°C until analysis.

### **Recording of the pharmacodynamic effect**

The antihistaminic effects of fexofenadine and cetirizine were recorded using an intradermal test (Fig. 10). The horses were shaved with an electric clipper on the lateral neck prior to intra-dermal injections with 7µg/site of histamine hydrochloride (0.1 mg/ml, ex tempore, Apoteket AB) using 27-gauge needles. The diameter of the skin reaction was measured after 20 min using a vernier calliper. Histamine-injections before administration of antihistamines served as positive controls. Sterile saline (0.07 ml) served as negative controls. Histamine-injections were then performed at different time points. The diameters of each wheal were determined by taking the mean of two perpendicular diameters. The wheal areas were then calculated and the antihistaminic effect was expressed as percentage inhibition of wheal formation compared to controls. Thus, for each horse the effect of the antihistamine on wheal areas were calculated by comparing the effect of histamine before and after administration of the drug. 0% inhibition was defined as the wheal area after the histamine injection alone (before administration of antihistamine) and 100% inhibition as the wheal area after injection of sterile saline. In paper II one to three injections were performed at each occasion and the

mean value was used for further calculations. The negative control value was calculated from at least 5 administrations for each horse. In paper IV three injections were performed at each occasion and the median value was used for further calculations. The negative control value was calculated from at least 11 administrations for each horse.



*Figure 10.* Histamine-induced cutaneous wheal formation in horse. Photo: Gunnar Carlsson.

### **Analysis of antihistamines**

In paper II the plasma concentrations of fexofenadine were quantified by liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS/MS) by use of a liquid chromatograph with a binary pump, a quadrupole-hexapole-quadrupole mass spectrometer with an electrospray interface and a Luna C8 chromatographic column. In papers III and IV the plasma concentrations of cetirizine were quantified by LC-ESI-MS/MS in a similar way as mentioned for fexofenadine. [ $^2\text{H}_6$ ]-fexofenadine (Toronto Research Chemicals Inc.) and [ $^2\text{H}_4$ ]-cetirizine (C/D/N Isotopes) were used as internal standards.

### **Analysis of PABA**

In paper V horse plasma was incubated with procaine (20  $\mu\text{g}/\text{ml}$  of plasma) in a water bath at 37°C for 12 min and the amount of PABA formed was measured by HPLC. Samples were then taken and deproteinised with acetonitrile and centrifuged at 12000  $g$  for 15 min. For determination of PABA 20  $\mu\text{l}$  of the supernatant were injected onto the HPLC. The endogenous PABA-levels in the plasma were determined separately and subtracted from the incubated samples.

### **Pharmacokinetic analyses**

For each horse the plasma concentrations of cetirizine were plotted versus time and the data were analysed using the software program Win Nonlin 2.0 or 5.0.1 (Pharsight Corporation). Different models and weighting factors were assessed by visual inspection of the curve fits and the residuals' scatter plots, and combined with the goodness of fit measures incorporated in the software, including the Akaike Information Criterion and the Schwartz criteria. These are statistical models that measure the trade off between the complexity of the model and how

well the model fits the data. They are used to decide the most suitable model when comparing the same data set and the same weighting scheme. The smallest Akaike and Schwartz values give the best fit for the models compared, whilst the most adequate weighing factor is measured by residual analysis.

In paper II a non-compartmental model was used for all data to compare the treatment groups (i.v and oral administration of fexofenadine with versus without ivermectin pre-treatment). The time ( $T_{max}$ ) to obtain the maximal plasma concentration ( $C_{max}$ ) and the  $C_{max}$  were read from the plotted concentration-time curve of each individual animal. The area under the curve (AUC) was calculated using the linear trapezoidal approximation. To extrapolate the AUC from time zero to infinity, the terminal slope ( $\lambda$ ) was used. Half-lives were determined from  $t_{1/2} = \ln 2/\lambda$ . The mean residence time (MRT) was calculated by the linear trapezoidal rule with extrapolation to infinity, using the formula:

$$MRT = AUMC / AUC,$$

where, AUMC is the area under the first moment curve.

The oral bioavailability ( $F$ ) was calculated from the AUC by using the equation:

$$F (\%) = 100 \times (AUC_{oral} \times dose_{iv}) / (AUC_{iv} \times dose_{oral})$$

The PK equation best fitted to use to the PK/PD link was a three-compartment model:

$$C(t) = I_1(e^{-k_1t} - e^{-k_1t^*}) + I_2(e^{-k_2t} - e^{-k_2t^*}) + I_3(e^{-k_3t} - e^{-k_3t^*})$$

where  $C$  is the plasma concentration at time  $t$  and  $t^*$  (the length of infusion) is included to create a factor that compensates for the time delay in relation to an i.v. bolus model (Fig. 11). The pharmacokinetic factors were calculated from the y axis intercepts ( $I_{1,3}$ ) and the exponents of the respective phase ( $k_1-3$ ). The weighting factor of choice was  $1/\text{the predicted Y-values}^2$  with iterative reweighting so that the weight redefines for each observation.

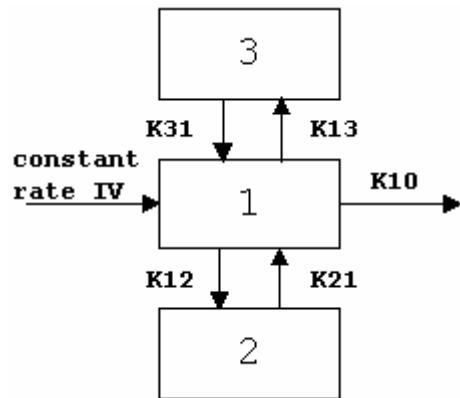


Figure 11. The three-compartment pharmacokinetic model with constant rate infusion. K12 represents the rate from compartment 1 to compartment 2, K21 the rate from compartment 2 to compartment 1, K13 represents the rate from compartment 1 to compartment 3, K31 the rate from compartment 3 to compartment 1, K10 the rate of elimination. Model from the WinNonLin library.

In papers III and IV the data were analysed and best fitted to a two-compartment model with first-order input, first-order output using the equation:

$$C(t) = A(e^{-\alpha t}) + B(e^{-\beta t}) + C(e^{-K_{01}t})$$

where  $C$  is the plasma concentration at time  $t$ ,  $K_{01}$  is the absorption rate and  $\alpha$  and  $\beta$  the declining rates (Fig. 12). The appropriate weighing factor was found to be  $1/Y$ , where  $Y$  is the observed concentration. The primary pharmacokinetic parameters were calculated from the y axis intercepts (A-C) and the exponents of the respective phase ( $\alpha$ ,  $\beta$  and  $K_{01}$ ). Secondary parameters calculated were the AUC from time zero to infinity, the  $T_{max}$ ,  $C_{max}$  and  $\alpha$  and  $\beta$  half-lives. For paper III the mean residence time (MRT) was calculated by the linear trapezoidal rule with extrapolation to infinity, using the formula:  $MRT = AUMC / AUC$ . For paper IV the  $T_{max}$ ,  $C_{max}$  for the second dose and the trough plasma concentration were read from the plotted concentration-time curve of each individual animal.

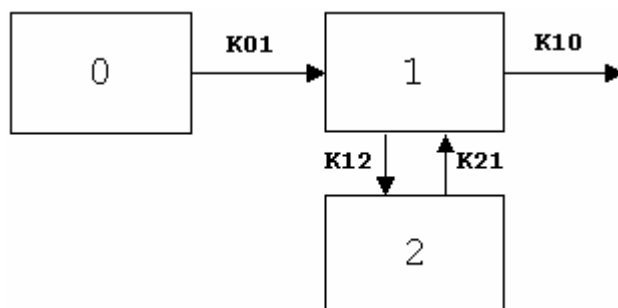


Figure 12. The two-compartment pharmacokinetic model with first-order input, first-order out-put.  $K_{01}$  represents the rate of the absorption,  $K_{12}$  the rate from compartment 1 to compartment 2,  $K_{21}$  the rate from compartment 2 to compartment 1,  $K_{10}$  the rate of elimination. Model from the WinNonLin library.

### Pharmacokinetic/pharmacodynamic link model

In papers II and IV a PK/PD link model was applied using pharmacodynamic data from a concentration/response equation and pharmacokinetic data from the three-respective two-compartmental models. Using the PK/PD link model, the maximal effect ( $E_{max}$ ), the plasma concentration producing 50 % of the maximal effect ( $EC_{50}$ ) and the rate constant for the elimination of drug from the effect compartment ( $K_{e0}$ ) were calculated (Toutain *et al.*, 1994, Toutain & Lees, 2004).

The concentration/response equation used was:

$$E = (E_{max} \times C_e^\gamma) / (C_e^\gamma + EC_{50}^\gamma)$$

where  $C_e$  is the apparent effect compartment concentration (Fig. 13). Gamma ( $\gamma$ ) is the slope factor which has its origin in the Hill coefficient and expresses the sigmoidicity of the concentration-effect relationship.

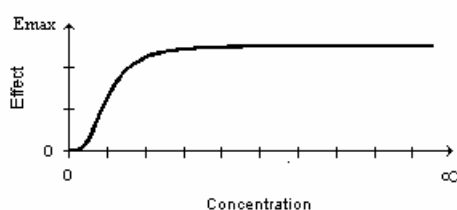


Figure 13. The Sigmoid  $E_{max}$  pharmacodynamic model describing the concentration and the response. Model from the WinNonLin library.



## **Statistical analysis**

In paper I a Pearson's product moment correlation was used. In paper II the pharmacokinetic data were subjected to statistical analyses using Student's t-test (paired, two-tailed test). For pharmacodynamic data an ANOVA was used followed by Dunnet's post-hoc test, comparing wheal areas before and after treatment. The significance level was set at 0.95 ( $p < 0.05$ ). For papers I and II, the software was Statview 4.0 (SAS Institute). In paper III pharmacokinetic data were subjected to statistical analyses using Student's t-test (paired, two-tailed test). In paper IV an ANOVA was used followed by Dunnet's post-hoc test, comparing wheal areas before and after treatment. The significance level was set at 0.95 ( $p < 0.05$ ). In paper V all plasma data were tested for normality with Anderson-Darling Normality Test before further analyses ( $p = 0.51$ ). Plasma data were subjected to statistical analyses using Student's t-test. The software was Minitab<sup>®</sup>, release 14 (Minitab Ltd.) for papers III, IV and V.

*De omnibus dubitandum est*

## Results and Discussion

### Occurrence and activity of CYP3A in the intestines (Paper I)

Real time RT-PCR analyses showed that the highest CYP3A mRNA expression was present in the duodenum with decreasing levels towards jejunum, ileum, caecum, and colon. The expression in the liver was similar to that in the anterior part of the jejunum, but about 4.5 times lower than in the anterior part of the duodenum. Immunohistochemistry showed CYP3A immunoreactivity in the cytoplasm of the enterocytes, which decreased distally along the intestinal tract. Studies in man, dog, rat, trout and mice have shown a similar pattern of intestinal mRNA expression and immunostaining (Bonkovsky *et al.*, 1985, Murray & Burke, 1995; Cotreau *et al.*, 2000; Kyokawa *et al.*, 2001; Lee *et al.*, 2001; Jin *et al.*, 2006) CYP3A dependent metabolic activity rose slightly from the anterior to the distal part of the duodenum and the anterior part of the jejunum and then declined to the middle and distal parts of the jejunum and the ileum, caecum, and colon. Studies in man have shown a similar pattern with a slight increase from the duodenum to the middle jejunum and then a decline towards the ileum. It has also been shown that the NADPH cytochrome P450 reductase activity exhibits the same pattern (Paine *et al.*, 1997). This may explain why there is no strict correlation between the mRNA expression and the CYP3A metabolic activity as the activity of CYP is dependent on the activity of NADPH cytochrome P450 reductase. Our results suggest that intestinal CYP3A in horses may limit the oral bioavailability of drugs and xenobiotics that are CYP3A substrates. CYP3A in the small intestine may play a major role in the first-pass metabolism and therefore affect the therapeutic efficacy of some drugs when administered orally in horses.

### Fate of the antihistamines and interactions with ivermectin (Paper II and III)

The PK of the histamine H<sub>1</sub>-antagonists fexofenadine and cetirizine and the effects of pre-treatment with the antiparasitic macrocyclic lactone ivermectin on the PK of the antihistamines were studied in horses. After intravenous infusion of fexofenadine at 0.7 mg/kg bw (paper II) the mean  $t_{1/2}$  was 2.4 h (range: 2.0-2.7 h), the apparent volume of distribution 0.8 L/kg (0.5-0.9 L/kg), and the total body clearance 0.8 L/h/kg (0.6-1.2 L/h/kg). The  $t_{1/2}$  after oral administration of fexofenadine was about 5.0 h, whereas the  $t_{1/2}$  after i.v. administration was considerably shorter. After oral administration the absorption rate can influence the rate of elimination (flip-flop phenomenon) and the apparent  $t_{1/2}$  may therefore increase (Baggot, 1992). After oral administration of fexofenadine at 10 mg/kg bw bioavailability was 2.6% (1.9-2.9%). The oral bioavailability of fexofenadine in mouse is also about 2%. The F in man has not been determined in detail, but appears to be higher than in horse and mouse (Lippert *et al.*, 1995; Krishna *et al.*, 2004; Thahara *et al.*, 2005). Oral therapy of horses with fexofenadine are not suitable due to the low bioavailability.

Ivermectin pre-treatment (0.2 mg/kg orally) 12 h before oral fexofenadine decreased the bioavailability to 1.5% (1.4-2.1%). In addition the area under the plasma concentration time curve decreased by 27%. Ivermectin did not affect the pharmacokinetics of i.v. administered fexofenadine. Ivermectin may therefore influence fexofenadine-absorption by interfering in intestinal efflux and influx pumps such as P-gp and OATP. Inhibition of the P-gp efflux pump would be expected to result in an increased uptake of fexofenadine, thus the opposite to the observed effect. Since several P-gp-inhibitors are also inhibitors of OATPs (Cvetkovic *et al.*, 1999) an alternative mechanism would be an ivermectin related inhibition of OATP-dependent influx pumps.

After oral administration of cetirizine at 0.2 mg/kg bw (paper III) the mean  $t_{1/2}$  was 3.4 h (range: 2.9-3.7 h) and the  $C_{max}$  132 ng/mL (101-196 ng/mL). The  $T_{max}$  was 0.7 h (0.5 – 0.8 h). The short  $T_{max}$  and absorption half-life (about 0.2 h) shows that cetirizine is rapidly absorbed in horses on an empty stomach. This property is often correlated to a rapid onset of action. The  $t_{1/2}$  was about 3.4 h, which indicates that steady-state plasma levels are attained by the first day if repeated doses are given. Ss is obtained after a delay of 3-5 times the  $t_{1/2}$  (for cetirizine 9-18.5 h) regardless of the dosing interval. Thus, for any drug having a  $t_{1/2}$  of 4.5 h or less the ss will be reached within the first day and a loading dose will not be required in this case. The  $t_{1/2}$  of cetirizine in horse is shorter than the  $t_{1/2}$  for humans, which is approximately 8 h (Gillard *et al.*, 2005; Peytavin *et al.*, 2005). Therefore administration twice daily would be appropriate in horse.

Ivermectin (0.2 mg/kg bw) given orally 1.5 h before cetirizine did not affect the pharmacokinetics of cetirizine. However, ivermectin pre-treatment 12 h before cetirizine increased the AUC by 60%. The  $C_{max}$ ,  $t_{1/2}$  and MRT also increased significantly following the 12 h pre-treatment. The  $T_{max}$  for orally administered ivermectin is about 3.6 h and the  $t_{1/2}$  about 3 days (Perez *et al.*, 2002). In this study the pre-treatment 1.5 h before cetirizine was assumed to mainly give a local effect on the enterocytes as the ivermectin concentration in the intestines should be high in the intestines and low in other tissues. The maximum systemic effects of ivermectin would not appear until after several hours. The pre-treatment 12 h before cetirizine was therefore assumed to have a more systemic effect also on the renal and bile excretion. This may explain why ivermectin affects the pharmacokinetics of cetirizine at the 12 h pre-treatment interval but not at the 1.5 h pre-treatment interval. We assume that the effect of ivermectin on the PK of cetirizine may be related to an inhibition of P-gp. A mechanism by which ivermectin might cause elevated plasma levels of cetirizine in horses would be increased intestinal uptake, related to inhibition of the P-gp efflux pump in the enterocytes. Ivermectin is eliminated mainly by transepithelial intestinal secretion (Laffont *et al.*, 2002). The enterocytes will therefore be exposed to high concentrations of ivermectin both during the absorption and excretion of the drug. Inhibition of the P-gp efflux pump would be expected to result in an increased uptake of cetirizine. The contribution of the intestinal P-gp to the overall absorption of cetirizine is difficult to elucidate without an i.v. study design. However since no effects on the PK of cetirizine were observed at the 1.5 ivermectin pre-treatment interval one may assume that the effect of ivermectin is

not primarily directed towards the intestinal cetirizine absorption. Instead the most important effect of ivermectin on the PK of cetirizine may be decreased renal excretion. Urinary excretion, which occurs both via active tubular secretion and glomerular filtration, is the major route of cetirizine elimination in humans (Benedetti *et al.*, 2001; Bree *et al.*, 2002). The elevated plasma levels may be due to decreased renal secretion and/or increased renal re-absorption of cetirizine, related to inhibition of the P-gp in the proximal tubular cells of the kidney. To discriminate if the effect of ivermectin is directed towards the P-gp activity at the renal and/or the intestinal level a study with intravenously administered cetirizine would be desirable (no such formulation was found in the market).

No side effects such as drowsiness or gastrointestinal disturbances were noticed for any of the horses used in this study. Cetirizine, as well as fexofenadine, is known to have insignificant or no anticholinergic effect (Orzechowski, Currie & Valancius, 2005; Liu & Farley, 2005). This is important for selection of drugs to be used in horses. The pharmacokinetic properties of cetirizine have characteristics that are suitable for an antihistamine and this substance may be a useful drug in horses.

## **Effects of the antihistamines and PK/PD modelling (Paper II and IV)**

The effects on inhibition of histamine-induced cutaneous wheal formation of the histamine H<sub>1</sub>-antagonist fexofenadine and cetirizine were studied in horses. After i.v. infusion of fexofenadine (paper II) at 0.7 mg/kg bw and after oral administration of fexofenadine at 10 mg/kg bw there were a significant decrease in histamine-induced wheal formation. After the i.v. administration the maximal effect peaked at 2 h with 70 % inhibition and remained over 45 % inhibition for 6 h. Significantly decreased values compared to controls were observed at 1, 2, 4 and 6 h (Dunnet's post-hoc test;  $p < 0.05$ ). After oral administration of fexofenadine the maximal effect peaked at 4 h with about 55 % inhibition. Significant differences compared to controls were observed at 4 and 6 h (Dunnet's post-hoc test;  $p < 0.05$ ). In man oral administration of fexofenadine at doses of 40 mg or higher inhibited wheal and flare reactions, induced by intracutaneous histamine provocation, for approximately 12 h (Russell, Stoltz & Weir, 1998) compared to the 6 h in horses. This could be explained if the  $t_{1/2}$  in horse is shorter than in man. Since different  $t_{1/2}$  have been reported in man - 3.5 h (Wang, *et al.*, 2002), 5.1 h (van Heeswijk *et al.*, 2006) 6.2 h (Uno *et al.*, 2006) and 10.1 h (Shimizu *et al.*, 2006) - it is difficult to draw any conclusions from this. Following the oral administration of fexofenadine in horse the  $C_{max}$  in plasma was about 86 ng/mL after dosing at 10 mg/kg bw. In man different  $C_{max}$  in serum has been reported: 160-450 ng/mL after a single oral dose of about 0.9 to 1.7 mg/kg bw of fexofenadine (Wang, *et al.*, 2002; Dresser, Kim & Bailey 2005; van Heeswijk *et al.*, 2006; Uno *et al.*, 2006; Shimizu *et al.*, 2006). The reported  $V_d/F$  in man after a single administration was calculated to be 5.6 to 8.9 L/kg (Wang, *et al.*, 2002; van Heeswijk *et al.*, 2006; Uno *et al.*, 2006; Shimizu *et al.*, 2006). The  $V_d/F$  (calculated as  $\text{dose}/\text{AUC} \cdot \lambda$ ) in horse when given 10 mg/kg orally was about 220 L/kg, whilst the  $V_{area}$  after i.v. fexofenadine was about 2.7 L/kg which is in the same magnitude

as in man. These data together indicate that there is a more effective absorption of fexofenadine in man compared to horses, even though the flip-flop phenomena contributes to the large oral V/F in horse. This allows lower therapeutic doses to be used in human medicine. A PK/PD link model indicated that fexofenadine in horse has antihistaminic effects at low plasma concentrations. In this model  $E_{max}$  showed a range between 55 and 97% and the plasma concentration required to reach  $EC_{50}$  was about 16 ng/mL. However, oral treatments of horses with fexofenadine may not be suitable due to the low bioavailability.

The PK and PD of cetirizine were studied following repeated oral administrations to the horses (paper IV). After three consecutive administrations of cetirizine (0.2 mg/kg body weight) every 12 h the trough plasma concentration of cetirizine was  $16 \pm 4$  ng/mL (mean  $\pm$  SD) and the wheal formation was inhibited by  $45 \pm 23$  %. Then the dose was increased to 0.4 mg/kg bw. After four additional administrations every 12 hours the trough plasma concentration was  $48 \pm 15$  ng/mL and the wheal formation was inhibited by  $68 \pm 11$ %. The maximal observed inhibition of wheal formation was  $84 \pm 7$ % which occurred 5 h after the last administration. Still 11 h after the last administration the inhibition remained over 55 % (Dunnett's post-hoc test,  $p < 0.05$  for 1, 3, 5, 7 and 11 h after last administration). The  $t_{1/2}$  was about 5.8 h. A PK/PD link model showed a high efficacy, the  $E_{max}$  was about 93% and the  $EC_{50}$  about 22 ng/mL. In humans Urien et al. (1999) showed an  $EC_{50}$  for wheal inhibition of about 40 ng/mL and an  $E_{max}$  of about 60% 3-5 h after an oral dose of 10 mg (approximately 1.4 mg/kg).

The PK calculations for cetirizine in horse showed that the AUC was 379 ng·h/mL (347-453 ng·h/mL) after an administration of 0.2 mg/kg bw. The  $t_{1/2}$  was about 5.8 h, the absorption  $t_{1/2}$  about 0.23 h and the  $T_{max}$  about 0.8 h. The short absorption  $t_{1/2}$  and the fast  $T_{max}$  show that cetirizine is rapidly absorbed in the horses. In paper IV cetirizine was given at 12 h-intervals to the horses mixed with sugar-beet and there was no restriction of the food-supply during the experiment. In our previous study (paper III) with the single dose of cetirizine the drug was given via a nasogastric tube and the horses were not fed 12 h before and 3.5 h after the administration (although they were bedded on straw). The results of paper IV showed that after a dose of 0.2 mg/kg bw the  $C_{max}$  was lower (58 ng/mL) than in the previous study (132 ng/mL), when cetirizine was given as a single dose (paper III). On the other hand similar values were observed in the two studies for the calculated AUC (379 and 420 ng·h/mL, respectively). In addition the calculated median terminal  $t_{1/2}$  in paper IV was longer (5.8 h) than in paper III (3.4 h). These data indicate that food present in the stomach and the proximal intestine will delay the absorption of cetirizine, but that the influence on the total extent of the absorption is of minor importance. Our results showed low inter-individual variability in plasma concentrations in the horses. This is a characteristic for drugs which have limited biotransformation. With reference to this observation one may assume that cetirizine in horse, like in man, is metabolised to a relatively low extent. This is advantageous for an antihistamine since it minimises metabolic drug-drug interactions. With regard to the observed half-life and the observed duration of the antihistaminic effect we consider that two daily administrations of cetirizine would be suitable in horse. Although the absolute bioavailability of

cetirizine was not assessed, our results indicate that the oral bioavailability in horses is sufficient, in contrast to the previously examined antihistamines clemastine and fexofenadine (Törneke *et al.*, 2003, Olsén *et al.*, 2006). The PK and PD properties of cetirizine have characteristics that are suitable for an antihistamine and this substance may be a useful drug in horses.

### **Adverse reactions to benzylpenicillin (Paper V)**

Case reports of 59 horses reacting adversely to benzylpenicillin in Sweden in 2003-2005 were obtained through contacts with horse-owners. For the assessment of the reports various parameters were evaluated such as the times to the reactions, information on previous penicillin treatment, the clinical signs and the actions taken in the reacting horses. Among the reports two horses had received sodium or potassium benzylpenicillin intravenously, whereas the remaining 57 horses had been treated with procaine benzylpenicillin intramuscularly. The dominance of reactions for procaine benzylpenicillin over the Na/K salts of benzylpenicillin correlates with previous reports in the literature (Marshall, 1980; Allpress & Heathcote, 1986; Nielsen *et al.*, 1988; Tjälve, 1997).

Allergy may underlie the adverse reactions in the horses given sodium and potassium benzylpenicillin and also in horses given procaine benzylpenicillin. However, in most horses in the latter group the clinical signs may be due to toxic effects of procaine. In these horses the dominating clinical signs were locomotor and behavioural changes. Some horses showing reactions for procaine benzylpenicillin received continued treatment with the same drug or with a Na/K benzylpenicillin salt without problems. It appears that penicillin allergy can be excluded in these cases.

Some risk factors may enhance the probability that horses react to procaine. Horses are more sensitive to procaine than humans (Tobin *et al.* 1977). This implies that some horses may be highly responsive to elevated plasma concentrations of procaine. Procaine benzylpenicillin was previously used to a considerable extent in human medicine. Acute adverse reactions were described in connection with such treatments (Hoigné's syndrome). The patients suddenly expressed extreme fear and had hallucinations and muscle tremor, but there were no blood pressure decrease or circulatory collapse. The symptoms appeared during or immediately following the administration of procaine benzylpenicillin and are probably caused by inadvertent intravascular penetration from the muscular injection site (Björnberg & Selstam, 1960; Downham *et al.*, 1978; Schreiber & Krieg, 2001). Following systemic administration procaine is a relatively specific stimulant of the limbic system, which in man can result in a range of emotional and somatic symptoms similar to those observed in Hoigné's syndrome (Servan-Schreiber *et al.* 1998). It is known that the limbic system is susceptible to neuronal sensitization, implying that there is a gradually increasing response after repetitive stimulation over time of sub threshold stimuli, which initially are without effects (kindling) (Adinoff *et al.* 2001). It has been proposed that a kindling mechanism, related to repeated injections of procaine benzylpenicillin, may increase the risk for Hoigné's syndrome (Araszkiewicz & Rybakowski, 1996/1997). It is possible that a

kindling mechanism may increase the susceptibility to procaine benzylpenicillin also in horse. In the present study 30 of the horses reacting to procaine benzylpenicillin had previously been treated with the same drug. It has been proposed that the mechanism of kindling may involve cross sensitization to local anaesthetics other than procaine (Araszkiewicz & Rybakowski, 1996/1997). It is possible that anaesthesia in horses with e.g. lidocaine in connection to previous surgical therapy or clinical examination for lameness might increase the sensitivity to procaine benzylpenicillin.

Procaine is rapidly hydrolysed by plasma esterases to the non-toxic metabolites PABA and diethylaminoethanol. When high amounts of procaine enter the circulation the hydrolysing capacity may be exceeded and toxicity occur. Analyses of plasma esterases from reacting horses showed lower activity than in non-reacting control horses. Thus, of the amount of PABA formed at the procaine hydrolysis in the group of reacting horses was  $7.13 \pm 1.54 \mu\text{g/mL}$  (mean  $\pm$  S.D.), whereas in the control group the amount of PABA formed was  $8.31 \pm 1.22 \mu\text{g/mL}$ . The difference between the two groups was statistically significant ( $p = 0.003$ ). Reduced plasma esterase activity may be one factor that increases the likelihood of adverse reactions by delaying the hydrolysis of procaine. It has been reported that a group of human patients who experienced systemic toxic reactions to procaine benzylpenicillin had significantly lower plasma esterase activity compared to a group of non-reacting patients (Downham *et al.*, 1978). It is concluded that several mechanisms may contribute to the adverse reactions of procaine benzylpenicillin in horses.

## Conclusions

- In horse intestines CYP3A mRNA is expressed to the highest extent in the proximal intestine, with a decreasing gradient toward the distal parts. The mRNA expression is higher in the proximal parts of the intestines than in the liver. CYP3A-enzyme activity increased slightly from the proximal to the distal duodenum and the proximal jejunum, followed by a plateau in the other parts of jejunum and a decrease towards ileum caecum, and colon. CYP3A-protein is localised intracellularly of the enterocytes. CYP3A in the small intestine may play a major role in the first-pass metabolism of drugs, which are substrates for these CYP-enzymes.
- Fexofenadine has antihistaminic effects at low plasma levels in horse. However, oral treatment of horses with fexofenadine is not suitable due to low bioavailability. The oral bioavailability of fexofenadine was significantly decreased when the horses were pre-treated with ivermectin before the fexofenadine administration. However, ivermectin did not affect the pharmacokinetics of i.v. administered fexofenadine. The mechanism underlying the effects of ivermectin on the pharmacokinetics of fexofenadine is unclear, but may be related to inhibition of the OATP influx pump in the intestinal mucosa.
- The pharmacokinetic and pharmacodynamic properties of cetirizine in horse have characteristics that are suitable for an antihistamine. The maximal inhibition of histamine-induced wheal formation was satisfactory and long lasting. Oral administrations of cetirizine in doses of 0.2-0.4 mg/kg given at 12 h intervals would be adequate to exert significant antihistaminic effects and this drug may be a useful antihistamine in equine medicine. Ivermectin administered at a 12 h pre-treatment interval, resulted in increased AUC,  $C_{max}$ ,  $t_{1/2\beta}$  and MRT of cetirizine in the horses. This effect may mainly be related to decreased renal excretion of cetirizine due to inhibition of P-gp in the proximal tubuli of the kidney.
- Acute adverse reactions to benzylpenicillin were much more frequent in horses treated with procaine benzylpenicillin than with potassium or sodium benzylpenicillin. The dominating clinical signs in horses reacting to procaine benzylpenicillin were locomotor and behavioural changes, which occurred during the injections or within one minute after the injections. Allergy may underlie some of the adverse reactions, but in the majority of cases the clinical signs may be due to toxic effects of procaine. Several mechanisms may contribute to the toxicity of procaine. Low plasma esterase activity may increase the likelihood for procaine toxicity and constitute one risk factor.



## Future perspectives

The results of this thesis indicate that in horse the intestinal mucosa is an important site of first-pass metabolism of drugs and other xenobiotics due to the high levels of CYP3A-enzymes in this tissue. Further studies to explore the consequences of CYP3A-related metabolism in the horse intestines on the disposition of drugs would be of interest.

It appears that there are only a few CYP-enzymes belonging to the families 1, 2 and 3 that are responsible for drug metabolism and thus involved in drug-drug interactions. It would be of interest to examine additional CYP-enzymes such as CYP2D6, CYP2E and also CYP1A and CYP2C in the intestines in horses.

It would be of interest to explore the occurrence of Phase II enzymes in the intestines of horse.

The result showed that cetirizine can be a useful antihistamine in horse. IgE and mastcell activation are involved in the pathogenesis of several disorders in horse, such as equine heaves, chronic bronchitis/bronchiolitis, chronic obstructive pulmonary disease/recurrent airway obstruction and the syndrome called “sweet itch”, “summer eczema” or “allergic urticaria” (a worldwide chronic seasonally recurrent pruritic allergic skin disease in horses). In addition IgE and mast cell activation are involved in allergic reactions to venoms and other antigens and in anaphylactic reactions to drugs. Extended studies to explore the therapeutic value of cetirizine in such conditions in horse would be of interest.

It is known that an atypical form of plasma esterase exists in humans (polymorphism). It would be of interest to examine if a similar polymorphism in procaine hydrolysing plasma esterases exists in horses, including studies on possible differences between different breeds.

It would be of interest to further explore the incidence by which allergy may underlie the adverse reactions to benzylpenicillin. For this purpose it would be valuable to have an allergy test against benzylpenicillin which can be applied to blood samples.

It would be of interest to do a follow-up study to see if there will be a declining number of adverse reactions to benzylpenicillin if benzylpenicillin derivatives with long duration but without procaine content will become available on the market. Such a formulation, consisting of benzylpenicillin with an ester-binding to diethylaminoethanol, is already available for cattle.

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## Populärvetenskaplig sammanfattning

Om hästen blir sjuk är det viktigt att den får rätt medicindos för att få bukt med sjukdomen och utan att den får biverkningar. Att förstå detaljerna om de funktioner som styr hur kroppen tar hand om ett läkemedel är viktigt både för den enskilda hästen och för arten. För att veta hur mycket och vilken slags medicin man ska ge till sin häst för att få den bästa effekten måste man veta hur läkemedlet verkar och hur hästar tar upp, bryter ned och utsöndrar de olika substanserna. Effekten av en medicin beror av hur kroppens organ och vävnader fungerar och samspelar när de påverkas av ett läkemedel.

Två viktiga faktorer som påverkar upptaget av medicinerna är enzymer och transportproteiner. I tarm och lever sker nedbrytningen med hjälp av metaboliska enzymer. Dessa enzymer avgör hur fort läkemedlet omsätts i kroppen. Vi har undersökt ett så kallat cytokrom P450 3A-enzym (CYP3A), var det finns, hur aktivt det är samt hur de gener som kodar för enzymet uttrycks. Transportproteinerna kan ”knuffa ut” medicinerna så att de inte kan verka i kroppen eller så kan de underlätta att medicinerna tas in i kroppen, så att de blir mer aktiva. I tarmen kan hastigheten för upptag och utsöndring av medicinen variera dels beroende av om man samtidigt äter mat eller något annat läkemedel dels beroende av olika transportproteiner och enzymer som sitter i tarmväggen. Transportproteinerna har också stor betydelse för hur medicinen förs från tarmen till blodet och vidare ut i hela kroppen. Både enzymer och transportproteiner påverkar därför tillsammans hur mycket medicin som kommer ut i hästens kropp. Ett sätt att undersöka detta är att mäta hur hög koncentration av medicin som finns i blodet. Vi har undersökt vad som händer om man påverkar effekten av ett transportprotein som kallas P-glykoprotein (P-gp) på upptaget av två olika läkemedel hos häst.

Det är dock inte bara enzymer och transportproteiner som påverkar effekten av olika mediciner och ibland kan hästarna drabbas av biverkningar som inte har direkt samband med de ovan nämnda faktorerna. Läkemedelsbiverkningar kan indelas i sidoeffekter, överdosedeffekter, allergier, förändringar i mikrofloran i mage och tarm, interaktioner (med andra läkemedel, mat, naturmedel mm), överkänslighet på grund av ärftliga orsaker (så kallad idiosynkrasi), lokala skadeeffekter, carcinogena effekter eller verkan på det ofödda fostret (teratogenicitet).

I samband med behandling av hästar med penicillinprokain förekommer att de drabbas av så kallad penicillinchock. Reaktionerna kan vara relativt lindriga, såsom hudreaktioner, eller att hästarna stelnar till under en kort stund, till allvarliga reaktioner då de ramlar ihop under kramper. Även dödsfall förekommer. Dessa reaktioner kommer oftast under injektionen eller strax efter avslutad injektion. Vi har sökt orsaken till varför behandling med penicillin kan ge chock hos hästar. Vi har undersökt hästarnas förmåga att bryta ned prokain. En dålig nedbrytningsförmåga kan leda till att prokainet blir en toxiskt (giftigt).

I den första delstudien i avhandlingen undersökte vi enzymet CYP3A i tarmen hos häst. Vi samlade in vävnadsprover från slaktmaterial. Genuttryck och enzymaktivitet studerades med olika biokemiska metoder. Vi tittade också på var och i vilka celler i tarmen enzymet finns. Detta undersökte vi med immunohistokemi. Det högsta uttrycket av CYP3A-genen fanns i början av tunntarmen och sedan sjönk nivån i riktning mot tjocktarmen. Aktiviteten hos enzymet följde i stort sett uttrycket av genen för enzymet i de olika delarna av tarmen. De celler som innehöll CYP3A fanns främst ytligt i slemhinnan i tarmväggen. Resultaten visar att läkemedel givna via munnen kan brytas ned av CYP3A redan i tarmcellerna.

I två andra delstudier har vi tittat på antihistaminer till häst. Användning av s.k. antihistaminer för att lindra allergier i framför allt övre luftvägarna hos människa är mycket utbredd. Antihistaminer används även till människor för att lindra klåda. Tidigare undersökningar av olika typer av antihistaminer till häst har visat att hästar inte tar upp dessa mediciner från mage/tarm speciellt bra. Ett bra antihistamin till häst ska vara effektivt och ta bort eller åtminstone dämpa de allergiska besvären. Det ska helst vara snabbverkande. Det ska ha en god biotillgänglighet, när man ger medicinen via munnen, det vill säga det ska tas upp i kroppen så att det kan göra verkan. Det får gärna vara verksamt under en lång tid så att man slipper medicinera flera gånger per dag. Det ska inte påverka eller påverkas av andra läkemedel. Det bör heller inte ha några svårare biverkningar. Man vill t ex inte ha en trött häst eller en som har problem med magen och får kolik. Syftet med dessa undersökningar var att hitta ett bra antihistamin som passar att ges via munnen till häst. Vi har tittat på två olika antihistaminer, fexofenadin och cetirizin, för att ta reda på om de går att använda till häst.

Allergiska problem kan vara ett användningsområde för antihistaminer till häst. Vid kontakt med främmande ämnen kan immunsystemet bilda en typ antikroppar som kallas immunglobulin av E-typ (IgE). IgE finns i mycket små mängder i kroppens vätskor och har förmåga att sätta sig fast på speciella celler såsom mastceller och vissa typer av vita blodkroppar. Mastcellerna finns utspridda i kroppens vävnader. Dessa celler innehåller ett flertal biologiskt aktiva ämnen som orsakar kraftig inflammation och klåda. Om en allergiframkallande substans (allergen; ämnet som utlöser bildning av IgE-antikroppar) kommer i kontakt med IgE-antikroppar och detta sker på mastcellens yta, frigörs aktiva ämnen från mastcellen såsom histamin. Lokalt uppstår då en kraftig inflammation med svullnad och rodnad i vävnaden. Histaminet som frisätts ger även en intensiv klåda.

Ett tillstånd som man anser ha samband med histamin och IgE-reaktion hos häst är ”sommareksem”. Sommareksem är ett stort problem för många hästar och deras ägare. Detta är en allergisk hudreaktion (dermatit) som ofta uppstår av allergi mot betten från svidknott (*Culicoides* spp). Även insektsbett från myggor eller allergiframkallande ämnen i luft och foder kan ligga bakom reaktionerna. Utvecklingen av allergi antas bero dels av moderns antikroppsöverföring till fölet, dels på genetisk disposition samt kraftig exponering för allergi-framkallande ämnen. För att hindra angrepp kan man försöka att hålla knotten borta genom att

ställa in hästarna, använda ”knottfria” beten (undvika låglänta och sankta marker) och/eller använda täcken. Som en del i detta kan också ingå att hålla hästen ren och använda insektsrepellerande medel. Det finns en mängd olika salvor, liniment och huskurer som används där vissa avser att hålla insekterna borta medan andra anses lindra eller förhindra uppkomst av inflammation men inga kontrollerade studier finns utförda. Ett läkemedel som används till häst för att minska inflammationen är kortison.

För att undersöka antihistaminerna gavs i våra undersökningar medicinerna till ett antal hästar. Blodprover togs med olika intervall både före det att hästarna fått medicinerna och flera gånger efter, oftast åtminstone upp till ett dygn efter medicineringen. Halterna av medicinerna i blodet mättes och korrelerades till den tid som förflutit sedan medicineringen. Effekterna av medicinerna mättes via ett ”pricktest”. Man ger då histamininjektioner in i huden. Då uppstår en kvaddel som är en hastigt uppträdande, välavgränsad röd upphöjning i huden. Kvaddeln uppkommer genom att vätska läckt ut från de små blodkärlen till bindväven. Vi mätte kvaddelarna och beräknade deras area. Som positiv kontroll användes histamininjektioner före medicineringen och som negativ kontroll användes fysiologisk koksalt.

I det ena försöket var det fem hästar som först fick en dos fexofenadin (Telfast) 10 mg/kg via munnen (oralt) och vid ett annat tillfälle fick de 0,7 mg/kg intravenöst (i.v.), i ett s.k. crossover-försök. Vi tog sedan upprepade blodprover och vi gjorde även histamintester efter medicin-givan. Resultaten visade att man fick en effekt (dvs en hämning av storleken av kvaddlarna) på 55% vid oral giva och detta varade i ca 4 timmar. Intravenöst var effekten 70% och den kvarstod i 6 timmar. Den högsta halten av fexofenadin uppmättes 1 timme och 15 min efter tillförelsen av fexofenadin. Då var koncentrationen fexofenadin i blodet 86 ng/ml. Halveringstiden (tiden som det tar att halvera halten i blodet) var 5 timmar för oral giva och 2,4 timmar för iv. Tyvärr var biotillgängligheten (dvs den mängd fexofenadin som togs upp i kroppen) väldigt låg, bara 2,6 %, vilket innebär att hästarna bara kunde tillgodogöra sig läkemedlet i mycket liten grad.

Vi gjorde då ett försök med cetirizin (Cetirizin). Detta försök hade ett lite annorlunda upplägg. Sex hästar fick flera givor av substansen; två gånger dagligen, 7 ggr totalt. Först fick de tre doser med 0,2 mg/kg; sedan dubblas dosen de sista 4 gångerna. Vi tog både blodprov och ”pricktest” var 12:e timme, precis innan de fick en ny medicindos. Detta innebär att vi mätte de lägsta blodnivåerna. Efter 3 doser med 0.2 mg/kg (efter 36 timmar) hämmades kvaddlarnas storlek med 45%. Koncentrationen i blodet var då 16 ng/ml. Efter ytterligare tre doser med 0.4 mg/kg (efter 72 timmar) hämmades kvaddlarnas storlek med 68%. Medicinkoncentrationen i blodet var då 48 ng/ml. Vi följde sedan upp med tätare prover efter sista dosen. Den största effekten kom 5 timmar senare och hämningen av kvaddelstorlekarna var då 84%. Vad gäller upptaget av cetirizin från tarmen så såg det också bra ut. Högsta halten uppmättes efter knappt en timme och var då 132 ng/ml. Halveringstiden var knappt 6 timmar. Sammanfattningsvis så verkar cetirizin vara ett bra antihistamin till häst. Hästarna tar upp tillräckligt av läkemedlet och det går bra att ge det i fodret. I doser på 0,2-0,4 mg/kg hindrar det

reaktionerna vid ”pricktest” med histamin i minst 11 timmar. Det bör därför ges två gånger dagligen för att vara så effektivt som möjligt.

I samband med undersökningarna med fexofenadin och cetirizin gjorde vi också studier av vad som händer om man påverkar transportproteinet P-gp. Vi vet sedan tidigare att både fexofenadin och cetirizin transporteras med hjälp av dessa proteiner. Vi ville ta reda på om läkemedlens omsättning i hästen kan förändras av att man förbehandlar eller samtidigt ger ett annat läkemedel som också transporteras av P-gp. Vi valde då ivermektin som är ett vanligt förekommande avmaskningsmedel till häst och som dessutom transporteras med hjälp av P-gp. Eftersom man kan anta att P-gp har fullt upp med att ta hand om ivermektin så bör halten av antihistamin i blodet bli påverkat om man har förbehandlat med ivermektin.

I den ena studien gav vi 0,2 mg/kg ivermectin (Noromectin) 12 timmar innan de fick fexofenadin. Med hjälp av blodprovsanalyser såg vi att hästarna tog upp fexofenadin ännu sämre än tidigare då de var förbehandlade med ivermectin. Det är oklart vad detta beror på. Det kan vara så att P-gp inte har så stor betydelse i det här fallet utan att andra transportproteiner som istället underlättar upptaget av fexofenadin över tarmslemhinnan har större betydelse.

I den andra studien fick hästarna också 0,2 mg/kg ivermektin, antingen 1,5 eller 12 timmar innan de fick cetirizin. Det visade sig att det inte blev någon effekt om man gav ivermectin 1,5 timmar före cetirizingivan. Däremot ökade cetirizin upptaget till blodet med 60 % om man gav ivermectin 12 timmar före cetirizin. Även om både koncentrationen i blodet och det totala upptaget till blodet ökade så såg vi inga oönskade effekter av cetirizinet, såsom t ex trötthet eller påverkan på tarmarna. Cetirizin är ett vanligt använt antihistamin till människa och där vet man att man kan ta flera gånger den rekommenderade dosen utan att man får biverkningar. Detta verkar gälla även för häst.

I den sista delstudien undersökte vi ”penicillinchock” hos häst. Penicillinbehandling av häst är mycket vanligt förekommande. Penicillin till häst ges alltid via spruta. Antingen ges ett vattenlösligt penicillinsalt, oftast i.v eller ett svårlösligt salt av penicillin och prokain i en muskel (i.m). Ofta görs medicineringen i en muskel av djurägaren eller en annan skötare. Att ge hästar penicillin via munnen som hos människa, hund och katt, är inte lämpligt eftersom hästar inte tar upp penicillin från tarmen till blodet speciellt bra och det finns även risk för att tarmfloran påverkas. Vanligtvis tål hästarna penicillin väldigt bra men i sällsynta fall kan de få en penicillinchock. Reaktionerna ses som regel efter i.m. injektioner av penicillinprokain, men ibland ses de även efter i.v. injektioner av de vattenlösliga penicillinsalterna. Hästarna reagerar i omedelbar anslutning till injektionerna med oro, svettningar och balansrubbingar och ibland ramlar de omkull. De återhämtar sig sedan oftast inom loppet av cirka en halvtimme, men i värsta fall kan de dö inom några minuter.

Det finns flera tänkbara mekanismer bakom dessa reaktioner. En möjlighet är att det är fråga om en allergi mot penicillin. För penicillinsalter som sprutats i.v. kan

man anta att allergi är orsaken. För penicillinprokain är det även möjligt att det är fråga om en toxisk reaktion mot prokain. Vid i.m. injektion av penicillinprokainet sker en långsam upplösning av saltet från muskeldepån.

Prokainets uppgift är att fördröja upptaget av penicillinet från injektionsplatsen. Utan prokain skulle man annars behöva spruta hästen var åttonde timma under behandlingsperioden. Med prokain räcker det med en dos dagligen. Om man vill ge ett vattenlösligt penicillinsalt ska det även ges direkt i blodet, vilket som regel kräver att hästen är inskriven på en klinik. Prokainet verkar också lokalbedövande på injektionsstället, vilket är positivt för hästen. Om preparatet skulle komma direkt ut i blodet kan det leda till skadliga effekter på grund av att halten fritt prokain i blodet blir för hög. Hästar är känsliga för prokain och man kan tänka sig att de reagerar p.g.a. prokaintoxicitet. Hästen kan då bli orolig, få svettningar och kramper. I normalfallet bryts prokainet ner till ofarliga beståndsdelar i blodet. Nedbrytningen av prokain sker med hjälp av enzymer, så kallade esteraser. En möjlighet som diskuteras är att vissa hästar skulle kunna ha en låg aktivitet av esteraser i blodet. Detta skulle kunna disponera dem för att drabbas av prokaintoxicitet. Det finns också beskrivet att man kan bli extra känslig för prokain (och andra lokalbedövningsmedel) efter upprepade injektioner.

Vi har tittat på 59 fall där hästar drabbats av någon form av reaktion vid behandling av penicillin. Hästarnas reaktioner varierade kraftigt - från ganska lindriga, såsom nässelutslag - till mycket våldsamma neurologiska reaktioner där hästarna slängde sig handlöst i boxen och tycktes vara helt okontaktbara. De flesta hästarna reagerade snabbt efter injektionerna. I studien har vi även undersökt esteraskiviteteten i blodet hos hästar som har fått en penicillinchock och jämfört dessa med hästar som inte har reagerat. Det visade sig då att hästar med låg esteraskivitet var betydligt överrepresenterade i chockgruppen. En dålig förmåga att bryta ned prokain kan vara en faktor som bidrar till att hästen har en ökad risk att drabbas av penicillinchock.

*Haec satis sunt dicta nobis*