Pharmacokinetics and Pharmacodynamics of Non-Steroidal Anti-Inflammatory Drugs in Birds

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ISBN 90-5864-033-7
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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Veterinary Science (PhD)

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<tr>
<td>5-HT</td>
<td>5-Hydroxy Tryptamine</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
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<tr>
<td>APP</td>
<td>Acute Phase Proteins</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
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<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>BW</td>
<td>Body Weight</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>Cl</td>
<td>Total Clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum Concentration</td>
</tr>
<tr>
<td>cMGF</td>
<td>chicken Myelomonocytic Growth Factor</td>
</tr>
<tr>
<td>COX1</td>
<td>Cyclo-Oxygenase 1</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclo-Oxygenase 2</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony Stimulating Factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>FLU</td>
<td>Flunixin</td>
</tr>
<tr>
<td>GA</td>
<td>Gentisic Acic</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
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<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
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<tr>
<td>HS</td>
<td>Hard Shelled</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IS</td>
<td>Internal Standard</td>
</tr>
<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>k&lt;sub&gt;el&lt;/sub&gt;</td>
<td>Elimination rate constant</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit Of Detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit Of Quantification</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>MEL</td>
<td>Meloxicam</td>
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</table>
MES  Morpholino-Ethane Sulfonic acid
MRL  Maximum Residue Limit
MRM  Multiple Reaction Monitoring
MRT  Mean Residence Time
MS   Mass Spectrometry
NMR  Nuclear Magnetic Resonance
NO   Nitric Oxide
NSAID Non-Steroidal Anti-Inflammatory Drug
OAA  Ortho-Anisic Acid
ODA  O-Dianisidine
PAA  Phenoxy Acetic Acid
PG   Prostaglandin
PIR  Piroxicam
RT   Retention Time
SA   Salicylic Acid
SC   Subcutaneous
SD   Standard Deviation
SL   Shell-less
SO   The ornithine conjugate of salicylic acid
SS   Soft Shelled
SU   Salicyluric Acid
$t_{1/2el}$ Half-life of elimination
$T_{max}$ Maximum Time
TNF  Tumor Necrosis Factor
TxB$_2$ Thromboxane B$_2$
UF   Ultrafiltration
UV   Ultraviolet
$V_{d(area)}$ Volume of Distribution (calculated via the area method)
WBC  White Blood Cells
Translated and adapted from:

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HISTORY OF NSAIDS

Salicylates in plant extracts have been in clinical use already centuries before Vane (1971) defined their principal mode of action as cyclo-oxygenase inhibitors. Hippocrates, Galen and early Greek and Roman herbalists have used medicinal extracts of barks which contained salicylates. The isolation of salicylic acid in the 19th century from willow and poplar barks led to its widespread use as an antipyretic, analgesic and anti-inflammatory agent. Since then, many non-steroidal compounds with anti-inflammatory properties have been discovered. The group is generally restricted only to those substances that act by inhibiting components of the enzyme system in the metabolism of arachidonic acid and formation of eicosanoids. These drugs may be structurally classified as carboxylic acids (R-COOH) or enolic acids (R-COH). The carboxylic acid derivatives include the salicylates (e.g. acetylsalicylic acid), acetic acids (e.g. indomethacin), propionic acids (e.g. ibuprofen), anthranilic acids (e.g. meclofenamic acid) and the aminonicotinic acids (e.g. flunixin). The enolic acids include the pyrazolones (e.g. phenylbutazone) and the oxicams (e.g. meloxicam) (Lees et al., 1991). New drugs are tested daily and may find their way to the market e.g. sulfonanilides (nimesulide) and coxibs (celexocib).

The use of non-steroidal anti-inflammatory drugs (NSAIDs) in veterinary medicine has followed the development and use of these drugs in human medicine. Since the early 70's, several NSAIDs and especially salicylates, as the oldest known NSAIDs, are routinely used in veterinary practice in the treatment of febrile states and inflammation disorders of several animal species, including cats, dogs, horses, chickens, swine, cattle (Yeary and Swanson, 1973 ; Mathur et al., 1974 ; Barber et al., 1974 ; Yeary and Brant, 1975 ; Fagot, 1975 ; Gingerich et al., 1975 ; Eyre et al., 1976). In recent years, the treatment of pain in animals is becoming an important issue, even in food producing animals. NSAIDs are routinely used in human medicine for the symptomatic treatment of pains of different origins. In food-producing birds as well as in companion birds, anti-inflammatory drugs are seldom prescribed. The main reasons are that no anti-inflammatory drugs are approved for use in birds and there is a scarcity of scientific research on inflammation, pain and NSAIDs in bird species. Nowadays, in food producing animals, the use of drugs is restricted to registered products for which a maximum residue limit (MRL) is established. After the administration of these drugs an appropriate withdrawal time must be applied before
slaughter and consumption of the animals. The legislator has listed these drugs in Annex I. The drugs that have a provisional MRL are listed in Annex III. The drugs that can be safely used in food producing animals and don't need an MRL are listed in Annex II. The drugs that are forbidden in food producing animals are listed in Annex IV (Anonymous, 2002). In the current legislation, a few NSAIDs have been assigned an MRL (Annex I) (carprofen (bovine, equine), vedaprofen (equine), flunixin (bovine, porcine, equine), tolfenamic acid (bovine, porcine), meloxicam (bovine, porcine)). One component has a provisional MRL (Annex III) that expires in July 2003 (metamizole (bovine, porcine, equine)). Two NSAIDs don't need an MRL (Annex II) (ketoprofen and salicylates) (Anonymous, 2002). Thus, these last two NSAIDs may be used in food producing species without withdrawal time before slaughter. However, according to the Belgian law, drugs that are not registered in Belgium cannot be administered to food producing animals. So, based on the current Belgian market, a few problems may arise. For example, no NSAIDs can be used in poultry industry, since there are no MRLs available for poultry, and the NSAIDs from Annex 2 are not registered in Belgium for these species. Registration procedures require, among other things, information on pharmacokinetics and pharmacodynamics of the drug in question in the species that will be treated with the drug.

The general information that is gathered about NSAIDS in this chapter is largely based on research in humans and different animal species, and where possible supplemented with bird-specific information. An important consideration is that fundamental data were described based on studies in mammals, and that it is possible that differences in inflammation and pharmacology of NSAIDs differ in birds and mammals.

PHARMACOKINETICS OF NSAIDS IN BIRDS

Few studies of the pharmacokinetics of non-steroidal anti-inflammatory drugs in birds have been published. The pharmacokinetics of ibuprofen in broiler chickens were investigated by Roder et al. (1996) and Vermeulen and Remon (2001). They report a mean elimination half-life of 2.7 h after intravenous (IV) administration and a low bioavailability of about 25% after oral administration. Large interindividual variations were present and these were attributed to delayed and incomplete absorption and enterohepatic recycling. An intravenous dose of 50 mg/kg resulted in central nervous
signs and death of the chicken. Indomethacin pharmacokinetics after oral and IV administration in poultry was studied by Cristòfol et al. (2000). A fast elimination half-life of 1 hour after IV administration was noticed. After oral administration the half-life was about 3 hours. The authors suggest a possible flip-flop phenomenon, due to unpredictable emptying time of the crop and the high pH of this part of the digestive tract. Flip-flop kinetics is observed when the rate of drug absorption process is so slow that it modifies the elimination rate from the body. Also a high variability between animals was observed after oral and even after intravenous administration.

PHARMACODYNAMICS OF NSAIDS

The generally accepted mechanism of action of NSAIDs is the inhibition of cyclo-oxygenase, an enzyme that converts arachidonic acid (AA) into eicosanoids (figure 1). Eicosanoids, such as prostaglandins and leukotrienes, are 20-carbon-chain derivatives of cell membranes. These compounds are synthesised when oxygen reacts with the polyunsaturated fatty acids of cell membrane phospholipids. The most important of these fatty acids is arachidonic acid (AA), which is released into the cell from damaged cell membranes. Once inside the cell, AA serves as a substrate for enzymes which generate eicosanoid products. Cyclo-oxygenases, located in all cells except mature red blood cells, add oxygen to AA, generating unstable prostaglandin endoperoxides. Subsequent peroxidase reactions result in the formation of prostaglandins and thromboxanes. Eicosanoids are potent mediators of inflammation (Boothe, 2001).

Two isoforms of cyclo-oxygenase exist: cyclo-oxygenase 1 (COX1) and cyclo-oxygenase 2 (COX2). COX1 mediates the formation of constitutive prostaglandins produced by many tissues, including gastrointestinal cells, platelets, endothelial cells and renal cells. Prostaglandins generated from COX1 are constantly present and impart a variety of normal physiologic effects: protection of gastrointestinal mucosa, hemostasis and maintenance of renal blood flow. COX2 catalyses the formation of inducible prostaglandins, which are only needed intermittently, e.g. during inflammation. NSAIDs appear to inhibit both COX1 and COX2. The amount of drug necessary to inhibit each of the two isoforms provides a basis for assessing the relative safety and efficacy of each drug. The ratio of COX2 to COX1 describes the amount of drug necessary to inhibit the respective isoforms of the cyclo-oxygenase
enzyme in an experimental environment. A COX2/COX1 ratio of less than 1 is desirable, since a drug that inhibits COX2 (inducible) prostaglandins at lower concentrations than that necessary to inhibit COX1 (constitutive) prostaglandins is probably safer. Salicylates, flunixin and phenylbutazone have a high COX2/COX1 ratio (preferential COX1 inhibitors) and meloxicam and carprofen are examples of NSAIDs with a favourable ratio (preferential COX2 inhibitors). New drugs are being developed with a specific action on the COX2 enzyme. Celecoxib and rofecoxib are specific COX2 inhibitors that are now available in human medicine (Boothe, 2001; Osiri and Moreland, 1999).

Figure 1. Sites of action of anti-inflammatory drugs on the arachidonic acid metabolism pathway.

Inhibition of cyclo-oxygenase as the sole anti-inflammatory mechanism of action of NSAIDs has been criticized. These drugs seem to alter cellular and humoral immune responses and may suppress inflammatory mediators other than prostaglandins. Several neutrophil functions may be inhibited, depending on the drug (Boothe, 2001).
For example, piroxicam inhibits both the generation of superoxide ions and the release of lysosomal enzymes, whereas ibuprofen does neither (Weissmann, 1991). Recent findings indicate that the COX2 enzyme is not only found after inflammatory stimuli, but also constitutively in brain, ovary, kidney and bone. Experiments with COX1 and COX2 deficient mice even show that both isoforms may act interchangeably in the absence of one. Other data indicate that COX2 is involved in the pathogenesis of Alzheimer disease and colorectal carcinoma (Osiri and Moreland, 1999).

**CLINICAL PHARMACOLOGY AND THERAPEUTIC USES OF NSAIDS IN BIRDS**

NSAIDs are commonly used in veterinary medicine for the treatment of inflammation of musculoskeletal and other tissues (e.g. spondylitis, laminitis, mastitis), endotoxic shock and colic in the horse and for the control of pain, associated with trauma or surgery (Boothe, 2001). These drugs may favourably influence the course and outcome of certain diseases and disorders. The ability of non-steroidal anti-inflammatory drugs to suppress inflammation and subsequent tissue damage is important, since the inflammatory process may result in organ damage that renders the animal unprofitable or useless for production (Kopcha and Alwynelle, 1989). Also the suppression of pain, which causes distress to the animal is an important pharmacological property of NSAIDs (Danbury et al., 2000). In bird medicine, a wide variety of indications exists for which treatment with NSAIDs could be beneficial (Bauck, 1990).

**Trauma**

When given post trauma and in cases of severe illness NSAIDs can be very useful. In ostriches, good results were found when used pre-operatively in reducing the pain response and promoting return to function, especially in leg surgery (Personal communication T. Gestier, 1998).

**Coccidiosis**

In broiler production systems, possible indications are respiratory diseases, digestive coccidial and bacterial infections and inadequate intestinal equilibrium to sustain good
weight gain (Cristòfol et al., 2000). In one trial, the effects of indomethacin (inhibits both COX1 and COX2) and nimesulide (a specific COX2 inhibitor) on an infection with coccidia were studied. No improvement on lesion scores or infection induced weight gain suppression was noticed, but treatment reduced oocyst output per chick with all indomethacin doses, and with the low nimesulide dose (Allen, 2000). In a recent trial with ibuprofen at an oral dose of 100 mg/kg, a significant reduction in oocyst shedding and intestinal coccidial lesions was seen (Vermeulen, 2002). A study about the pathogenesis of Cryptosporidium baileyi showed that a treatment with indomethacin reduced the excretion of oocysts and shortened the time of excretion (Hornok et al., 1999).

Sudden death syndrome or broiler ascites
Also sudden death syndrome in turkeys and heavy breed chickens has been suggested as indication for NSAID treatment. Experiments with dietary aspirin were done in both species, but no influence on sudden death syndrome could be found (Proudfoot and Hulan, 1983 ; Boulianne and Hunter, 1990). The etiology of broiler ascites, a similar disease, is still under investigation by several researchers. It seems that an imbalance between oxygen supply and the oxygen required to sustain rapid growth rates and high food efficiencies causes broiler ascites in chickens. There is an inadequacy of vascular capacity for blood flow through the lung to provide the tissues with the oxygen needed. This leads to pulmonary hypertension-induced ascites. Several factors can have an impact on the occurrence of the disease: partly genetic, metabolic (hypothyroid state, cardiac pathology) and environmental (diet, high altitude, drugs, infections), or the interaction between these factors (Decuypere et al., 2000 ; Julian, 2000). In another experiment, dietary aspirin was given to chickens kept in a hypobaric chamber, to mimic high altitude. The prostaglandin inhibitory effect of acetylsalicylic acid could promote vasodilatation and prevent blood clotting in broilers. A reduction in ascites was seen in aspirin treated birds (P ≤ 0.06), compared to controls, but also the smaller weight of these birds raised at high altitude could have caused the reduction in ascites incidence (Balog et al., 2000).

Heat stress
Several authors have investigated a supposed beneficial effect of salicylates on the growth and egg production of chickens during heat stress. One study in layer hens
shows an improvement on growth and size of eggs, when a combination of ascorbic acid (200 mg/kg) and aspirin (0.20%) was added in the food. No effect was noted on the body temperature (Oluyemi and Adebanjo, 1979). Another study in laying hens shows improvement in feed intake, egg production, eggshell weight and reduction of mortality. This was seen in the highest treatment groups (600-800 mg/kg feed) and after 3 weeks of supplementation (Abd-Ellah et al., 1997). Other researchers could not repeat this experiment and found no improvement at 0.20% aspirin in the feed. They even found a significant depression on egg production at higher levels (0.60%) (Mathur et al., 1974). Broiler chickens also seemed to have higher survival rates after treatment with flunixin during heat stress periods (Oliver and Birrenkott, 1981). However, Stillborn et al. (1988) found that dietary supplementation of aspirin provided little benefit to heat stressed broilers.

**Growth or egg production**

Another group studied hen performance and eggshell quality after chronic feeding of an aspirin added diet. No improvement in egg production or feed efficiency was found. In addition, early hen liveability and eggshell quality were worse in the aspirin fed chickens (McDaniel et al., 1993a). An older study, however, showed good results after a 0.05% dietary aspirin treatment with increased egg production and feed efficiency (Thomas et al., 1966). One group studied the short term effect of high doses of aspirin in the food of laying hens (4 -7.5 g/kg feed) and observed no change in investigated parameters (egg production, time of oviposition, behaviour of birds) (Gilbert et al., 1982).

A common avian reproductive dysfunction is the inability of hens to deposit shell adequately on eggs. Some of these soft-shelled (SS) and shell-less (SL) eggs are laid prematurely. It was hypothesised that prostaglandins may be responsible for the premature expulsion of some SS or SL eggs. But it was concluded that aspirin could only reduce the production of SL eggs in favour of the SS eggs. Probably due to physiological limitations in calcium mobilisation, SL eggs could not be converted into hard-shelled (HS) eggs (Balog and Hester, 1991). Also, the fertility and hatchability of embryos exposed to elevated incubation temperatures was investigated with and without administration of aspirin to layer breeders. No differences in chick hatchability were found (McDaniel et al., 1993b).
Locomotion disturbances

Also locomotion disturbances are a possible indication for using NSAIDs. Aspirin is used frequently in duck production when a rheovirus infection with tenosynovitis occurs. Also in turkey industry aspirin is used in certain leg problems (Jouglar and Benard, 1992). Degeneration of the hip and other joints is prevalent in male breeding turkeys at the end of their breeding life (Hocking, 1988) and there is evidence that affected birds experience pain during locomotion. The major effect of an anti-inflammatory agent (betamethasone) was to increase measures of behaviour associated with activity. This can be measured by a pedometer or the time needed to get to a food and drinking bowl (Duncan et al., 1991, Mc Geown et al, 1999). In rapidly growing broiler chickens the same pathology exists, and is defined as a non-infectious cause of lameness. Treatment with carprofen has proven to increase the walking speed of affected chickens, providing evidence that birds with moderate lameness suffer pain when they walk (Mc Geown et al., 1999). Furthermore, self-selection of analgesic agents is a well validated model for investigating chronic pain in animals, and Danbury et al. (1997) have shown that lame broiler chickens self-select more feed containing an analgesic agent than sound birds. An experimental inflammation protocol has been developed by Hocking et al. (1997) to determine the effectiveness of different analgesics for reducing articular pain in domestic fowl. Arthritis was induced by sodium urate in the hock joint and behavioural profile was studied. Bupivacaine at a dose of 3 mg was able to restore behavioural profile of the birds. Different opioid agonists, however, with a high affinity for the µ receptor, could not alter the pain behaviour after the inflammatory stimulus (Gentle et al., 1999). Broiler breeder males with lesions walked more slowly when they were given an injection of naloxone, an opioid antagonist. This was the first evidence that an endogenous system of analgesia exists in the fowl. Subsequent work has demonstrated that a powerful system of endogenous analgesia operates in the chicken in response to specific environmental stimuli (Gentle and Corr, 1995) and it was considered possible that the incentive of food or a sexual encounter might stimulate this endogenous analgesia in birds with leg lesions. The sodium urate-induced arthritis model was further used in chickens to study the effects of changes in attention (a novel pen) on pain-coping behaviour and inflammation. The authors suggest that changes in attention cannot only reduce the pain of arthritis, but also the peripheral inflammation,
since they have seen a less elevated skin temperature over the inflamed joint (Gentle and Tilston, 1999).

**Pain related to beak trimming**

Another possible use could be the analgesic therapy of beak-trimmed chickens. It is known that beak trimming excites nociceptors in the beak leading to short-term acute pain and a reduction in feed intake (Glatz *et al.*, 1992, Hughes and Gentle, 1995). Already experiments with a combination of bupivacaine and dimethyl sulfoxide have shown that application of this mixture on the wound can maintain the feed intake the first day after trimming. This probably indicates that some of the acute pain had been relieved (Glatz *et al.*, 1992).

**ADVERSE REACTIONS OF NSAIDS**

The majority of adverse reactions reflects the inhibitory effects of NSAIDs on prostaglandin activity. In addition, combination of several NSAIDs can be fatal. The major toxicities affect the gastro-intestinal, hematopoietic and renal systems. Miscellaneous effects associated with use of NSAIDs include hepatotoxicity, aseptic meningitis, diarrhoea and central nervous system depression (Boothe, 1989).

Gastrointestinal erosions and ulcerations are the most common and serious side effects of the NSAIDs. Inhibition of prostaglandin E₂-mediated bicarbonate and mucus secretion and blood flow seems to be the mechanism. Direct irritation of acidic drugs may be important and impaired platelet function may contribute to mucosal bleeding. Toxicity of an NSAID can be species dependent e.g. ibuprofen is relatively safe in humans, but in dogs serious gastro-intestinal side effects can occur.

All NSAIDs are able to impair platelet activity due to impaired thromboxane synthesis. Aspirin irreversibly acetylates the platelet cyclo-oxygenase. Since platelets cannot regenerate cyclo-oxygenases, platelet aggregation defects caused by aspirin can last up to 1 week.

Renal toxicities are mainly caused by NSAID inhibition of COX1 enzyme. These include renal vasoconstriction and renal insufficiency. This nephropathy does not occur frequently in domestic animals, but patients suffering from cardiac, liver or renal diseases, hypovolemic patients and patients receiving nephrotoxic drugs are predisposed (Boothe, 2001).
CONCLUSION

In birds a lot of the basic knowledge on drugs and inflammation is still lacking. No pharmacokinetic data of veterinary relevant NSAIDs exists. Many of the possible indications are empirical or based on the general mechanism of action of NSAIDs and treatment results are often contradictory and not reproducible. The need for a better understanding of the different mechanisms involved in inflammation in birds, the mechanism of action and the pharmacokinetics of NSAIDs in birds is evident.
REFERENCES


Non-steroidal anti-inflammatory drugs have been used in mammals for many decades. Salicylic acid was first synthesised in 1859 and marketed in 1878 and was the first drug with recognised antipyretic, anti-inflammatory and analgesic capacities. Since then, many new drugs with similar activities were developed. The pharmacological knowledge of the NSAIDs is mainly limited to mammal species. Therefore, the scientific aims were to investigate both the pharmacokinetic and pharmacodynamic aspects of these drugs in birds.

Pharmacokinetic aspects:

1. The investigation of the intravenous pharmacokinetic behaviour of sodium salicylate, flunixin and meloxicam in five food producing bird species (chickens, turkeys, ostriches, ducks and pigeons).

2. The investigation of the amino acid conjugation excretion pattern of sodium salicylate in chickens and pigeons.

Pharmacodynamic aspects:

1. The development of a subcutane inflammation model in chickens to investigate several inflammation parameters after treatment with sodium salicylate.

2. The development of an intravenous lipopolysaccharide model in chickens to investigate the acute phase reaction after treatment with sodium salicylate.
Pharmacokinetics of NSAIDs in birds

'Pharmacokinetics is what the body does to the drug'
Different aspects of pharmacokinetics in birds

Translated and adapted from:

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ABSTRACT

The study of the pharmacokinetics of drugs administered to animals provides information about the drug passage (absorption, distribution and elimination) within the body. Area under the curve, volume of distribution, clearance and half-life of elimination are parameters that allow the pharmacologist to determine if the drug reaches its site of action, to what extent it has arrived, and how long it remains at the site of action.

In this chapter, the general principles of pharmacokinetics are explained and relevant information about the metabolism of salicylic acid, the current knowledge about pharmacokinetics of NSAIDs in birds and some facts about the anatomy and physiology of birds are provided.
INTRODUCTION

Pharmacokinetics is concerned with the study and characterisation of the time course of drug absorption, distribution and excretion. Additionally, it studies the relationship of these processes with regards to the intensity and duration of characteristic effects of drugs (Baggot, 1995). An understanding of the dose-effect relationship can generally be obtained by linking pharmacokinetic behaviour with information on pharmacodynamic activity (Holford and Sheiner, 1981). To produce its characteristic effect, a drug must attain effective concentrations at its site of action. In veterinary medicine, this requirement is complicated by the variety of animal species to which therapeutic agents are administered. Wide variations in intensity and duration of pharmacologic effect are commonly observed among species of domestic animals when a drug is given at a similar dose. These variations in response can be attributed to species differences either in the availability of the drug or in the inherent sensitivity of tissue receptor sites (Baggot, 1995). Clinical pharmacological studies mainly support the view that species variations in response to drugs are attributed to differences in one of the following processes: systemic availability, accessibility to the site of action and the rate of elimination, including biotransformation and excretion (Dorrestein, 1991). When a drug is administered by the oral route, the rate and extent of its absorption from the gastro-intestinal (GI) tract are likely to vary between the species. Some anatomical features of the gastro-intestinal and renal system in birds are discussed further in this chapter.

PHARMACOKINETICS : GENERAL PRINCIPLES

Drug administration and bioavailability

A drug can be given either orally or by a parenteral route when systemic effects are desired. Orally administered drugs must undergo some events before entering the systemic circulation: release from the dosage form, transport across the GI mucosal barrier, and passage through the liver. Parenteral administration indicates that the GI tract is bypassed and the drug is given by injection, topically or via inhalation. The major routes of parenteral administration are intravenous (IV), intramuscular (IM) or subcutaneous (SC). Parenteral injection necessitates that strict asepsis be maintained to avoid infection. A variable degree of drug absorption takes place from these sites of
administration, the extent of which depends largely on the formulation of the administered preparation and on the drug itself. A low degree of ionization and high lipid solubility of the non-ionized moiety are properties favouring absorption (Baggot, 1995). The injection of a drug solution directly into the bloodstream gives a predictable concentration of the drug in plasma. A unique feature of the IV administration is that the drug is completely available systemically. Bioavailability is defined as the rate and extent to which a drug, administered as a particular dosage form, enters the systemic circulation intact. Bioavailability of a drug can be adequately characterised by determining three parameters from the plasma drug concentration-time profile: peak plasma concentration ($C_{\text{max}}$), time taken to reach peak concentration ($T_{\text{max}}$), and area under the curve (AUC). Considerable variations in bioavailability of drugs from oral dosage forms are likely to exist between species (Rowland and Tozer, 1995).

**Rate of drug movement**

Pharmacokinetics may be defined as the mathematical description of changes in drug concentration in the body. These changes can obey first order or zero order rates. In first order processes, the actual rate of the process varies in direct proportion to the mass of the compound. In zero order processes, the rate of drug movement is fixed and thus independent of the amount of compound available (Riviere, 1999). First order kinetics is typical in most drug studies. The elimination of a drug by saturable processes is described by Michaelis-Menten kinetics. At low drug concentrations the drug follows first order kinetics. When the elimination processes become gradually more saturated, the drug follows mixed order kinetics, eventually discharging into zero order kinetics, at high drug concentrations (Shargell and Yu, 1993).

**Disposition curve and compartment models**

Following an IV injection of a single dose of the drug, the decline in plasma concentration of the drug is expressed graphically by the disposition curve. This curve, plotted on semilogarithmic graph paper, is either monophasic (figure 1) or biphasic (figure 2). Compartment analysis, in which the body is conceived as consisting of distribution compartments interconnected by first order rate constants defining drug transfer, is used to describe the pharmacokinetic behaviour of drugs. Usually these compartments, which are mathematical entities, have no physiologic
counterpart. The models are open compartment models, since there is elimination of the drugs. Elimination is assumed to take place exclusively from the central compartment. Furthermore, distribution and elimination processes associated with the model are assumed to obey first order kinetics (Riviere, 1999).

Figure 1. Semilogarithmic graph showing first-order (mono-exponential) decline in plasma concentration of a drug with time (Baggot, 1995).
Figure 2. Semilogarithmic graph showing first-order (bi-exponential) decline in plasma concentration of a drug with time (Baggot, 1995).

The drug is introduced into the central compartment, where it equilibrates almost instantaneously. For many drugs, the central compartment consists of the blood and tissues of highly perfused organs such as lungs, liver and kidneys. Distribution between the central and the peripheral compartments takes place more slowly. The peripheral compartment may be considered to consist of less well perfused tissues such as muscle, skin and other less perfused organs (figure 3).
One compartment model

Two compartment model

Figure 3. Schematic diagram of the one- and the two-compartment open model. The drug is introduced in and eliminated from the central compartment. $k_{12}$ and $k_{21}$ are first order rate constants for drug transfer between the two compartments and $k_{el}$ is the first order rate constant for drug elimination from the central compartment (Baggot, 1995).

The following equations are used to describe the concentration-time curves of drugs administered after intravenous administration: a mono-exponential equation that describes a one compartment open model $C = C(0)e^{-kt}$ and a bi-exponential equation that describes a two compartment open model $C = A_1e^{-\alpha t} + A_2e^{-\beta t}$. In these equations $C$ represents the plasma concentration, $C(0)$ is the extrapolated initial concentration, $k$ is the elimination rate constant, $A_1$ and $A_2$ are mathematical coefficients, $\alpha$ represents the distribution rate constant, $\beta$ is the elimination rate constant and $t$ is the time (Gibaldi and Perrier, 1975).

**Half-life, volume of distribution and clearance**

The rate of drug elimination is determined mainly by the elimination mechanisms. The half-life of elimination ($t_{1/2el}$) of a drug is defined as the time required for the body to eliminate one-half of the drug. The half-life is calculated simply by measuring the time required for any given plasma concentration of the drug to decline by 50% during the linear terminal phase of the drug concentration-time profile. If the drug obeys first order kinetics, the half-life value is independent of the dose administered.
When drug absorption from the GI tract or an injection site is rapid, the half-life is independent of the route of administration. However, IV injection of a single dose is the only satisfactory procedure for the calculation of the half-life. When drug elimination obeys zero-order kinetics, the half-life becomes progressively longer as the dose is increased. The usual cause of dose-dependent elimination is the limited capacity of certain drug metabolising enzyme systems (Baggot, 1995). An example of zero-order is the dose-dependent half-life of salicylate in cats, due to the impaired microsomal glucuronyl transferase activity in cats (Yeary and Swanson, 1973).

An estimate of the extent of distribution of a drug is given by the apparent volume of distribution \( V_{d(area)} \). This value serves as a proportionality constant relating the total amount of drug in the body at any time to the plasma concentration of a drug after pseudodistribution equilibrium has been attained. It is the volume of fluid that would be required to contain the amount of drug in the body if it were uniformly distributed at a concentration equal to that in the plasma. This parameter does not distinguish between widespread distribution and high affinity binding with restricted distribution.

The total clearance (Cl) is a good index of efficiency of drug elimination. It represents the sum of clearances of the drug by the elimination organs (liver, kidney, lung, etc.). It is defined as the volume of plasma cleared of the drug by various elimination processes per unit of time (Shargel and Yu, 1993).

**Mean residence time**

After the administration of a dose of a drug, a large number of drug molecules distribute throughout the body. These molecules will stay (reside) in the body for various time periods. Some drug molecules will leave the body almost immediately after entering, whereas other drug molecules will leave the body at later time periods. The term mean residence time (MRT) describes the average time for all the drug molecules to reside in the body. MRT may also be considered as the mean transit time.

**Plasma protein binding**

Binding of a drug to plasma proteins restricts its distribution and can influence the elimination of the drug from the body. The main plasma protein to which the drugs bind is albumin. Protein binding is a reversible interaction and it is well known that only the lipid soluble, non-ionized moiety of a drug that is free (unbound) in the
plasma can penetrate cell membranes or diffuse into transcellular fluids. The drug binding is usually classified as extensive (>80%), moderate (50-80%) and low (<50%) (Riviere, 1999).

DRUG ELIMINATION

Mechanisms of drug elimination
Biotransformation and excretion are the two mechanisms of drug elimination. Hepatic metabolism and renal excretion are involved in elimination of most drugs. Apart from the liver, metabolism of drugs can also take place in blood, lung, kidney, lumen of the gut, etc. Apart from the kidney, excretion of drugs can also occur in the bile, lung, sweat glands, etc. Biotransformation decreases the lipid solubility of drugs, so their metabolites are readily excreted. Polar drugs and compounds with low lipid solubility are eliminated mainly by excretion (Baggot, 1995).

Biotransformation
Biotransformation is the formation of metabolites that have physicochemical properties favourable to their excretion. The general pattern of drug metabolism is usually biphasic. The first phase (Phase I) consists of reactions that can be classified as oxidative, reductive and hydrolytic, while the second phase (Phase II) includes the synthetic reactions (conjugations) (figure 4). Phase I transformations usually introduce polar groups into the molecule, which can then undergo conjugation with endogenous substances such as glucuronic acid, acetate, sulfate and various amino acids (Williams, 1967).

![Figure 4. The general pattern of drug metabolism (Williams, 1967).](image-url)
The nature of the amino acid involved in the conjugation reactions depends both on the animal species and on the chemical structure of the drug. In general, the amino acids participating in conjugation reactions are glycine and to a lesser extent glutamine, taurine and ornithine (Kasuya et al., 1999). There have only been a few reports on ornithine conjugation and the authors indicated that ornithine conjugation is restricted in the species occurrence. It has been found that ornithine conjugation occurs only in some birds and reptilian species but has not been reported in mammalian species (Igarashi et al., 1992). Based on the fact that birds possess arginase in the kidney, a function of arginase may be to supply ornithine for detoxification. Dietary arginine may be a source of ornithine for conjugation reactions in birds (Seymour et al., 1987).

**Excretion**

Excretion in the kidneys is complex and involves the following mechanisms, depending on the physicochemical properties of the drug: glomerular filtration of unbound drugs, carrier mediated excretion by the proximal tubular cells and passive reabsorption in the distal portion of the nephron (Shargel and Yu, 1993; Rowland and Tozer, 1995).

The excretion of drugs into the bile depends mainly on the particular substance and to some extent on species, which may be grouped together as good (rats, dogs, chickens), moderate (cats, sheep) and poor (guinea pigs, rabbits, rhesus monkeys) biliary excretors (Williams, 1967). After entering the small intestine via the bile, some drugs are reabsorbed or the glucuronide conjugate is hydrolysed and the parent drug is reabsorbed. This cycle is known as the enterohepatic circulation of a drug. This phenomenon can increase the half-life of a drug that is eliminated by renal excretion (Riviere, 1999).

**General scheme of salicylic acid biotransformation**

The biotransformation pattern of salicylic acid has been studied extensively in humans. This information was the starting point of the research of the metabolism of salicylic acid in other species. The different metabolism pathways of salicylic acid in humans are shown in figure 5.
Figure 5. Metabolic pathways of salicylic acid (SA) in human. Pathways indicated with solid lines follow first order kinetics whereas those indicated with dashed lines are saturable (Michaelis-Menten) pathways. SAG: salicyl acyl glucuronide; SPG: salicyl phenolic glucuronide; SU: salicyluric acid (the glycine conjugate); SUPG: salicyluric phenolic glucuronide; GU: gentisuric acid; GA: gentisic acid (Shen et al., 1991).

PHARMACOKINETICS OF NSAIDS IN BIRDS

The NSAIDs share a number of pharmacokinetic properties. As weak acids, they tend to be well-absorbed following oral administration. Food can impair the oral absorption of some NSAIDs. The drugs are lipid soluble but are characterised by a small volume of distribution due to binding to serum albumin, which can exceed 99% for some compounds in some species. Clearance of the NSAIDs is variable, differing
among drugs and species. Most NSAIDs are eliminated primarily after hepatic metabolism. Both phase I and phase II hepatic drug-metabolising reactions are important. Age and species differences in drug clearance should lead to caution when extrapolating doses from one animal to another (Boothe, 1995). Few studies of the pharmacokinetics of non-steroidal anti-inflammatory drugs in birds have been published. The pharmacokinetics of ibuprofen in broiler chickens was investigated by Roder et al. (1996) and Vermeulen and Remon (2001). The indomethacin pharmacokinetics after oral and intravenous administration in poultry was studied by Cristófol et al. (2000).

ANATOMY AND PHYSIOLOGY OF BIRDS

Digestive system
The digestive system has adaptations designed to facilitate flight. The length of the intestinal tract is shorter in birds relative to mammals. Also, birds lack teeth and heavy jaw muscles which have been replaced with a light-weight bill or beak. Food particles are swallowed entirely into the crop and after passage through the proventriculus, are reduced in size by the ventriculus or gizzard located within the body cavity (Denbow, 2000). The digestive tract of a 12-week-old turkey is shown in figure 6.

There are many species variations, e.g. ostriches do not have a crop, but have an especially large proventriculus, in pigeons the ceca are rudimentary, while chickens and turkeys have a pair of well developed ceca, and while most species have a gall bladder, pigeons and ostriches do not have one. The species variations have been described by McLelland (1975). Because of marked differences in the digestive system of different species of birds, variations can be expected in both rate and extent of drug absorption from an oral preparation. The crop has a storage function and depending on the quantity of the food accumulated in the crop, the emptying of the crop can be delayed several hours. Also the strong acidic content of the proventriculus and the mechanical action of the gizzard can play a role. However, a stable drug solution should pass quickly through the crop, oesophagus and stomach and should be available for absorption from the intestine within minutes of administration (Dorrestein and Van Miert, 1988).
Figure 6. The digestive tract of a 12-week-old turkey (1 precrop esophagus, 2 crop, 3 postcrop esophagus, 4 proventriculus, 5 isthmus, 6 thin craniodorsal muscle, 7 thick cranioventral muscle, 8 thick caudodorsal muscle, 9 thin caudoventral muscle (6-9 gizzard), 10 proximal duodenum, 11 pancreas, 12 distal duodenum, 13 liver, 14 gallbladder, 15 ileum, 16 Meckel's diverticulum, 17 ileoceccolic junction, 18 ceca, 19 rectum, 20 bursa of Fabricius, 21 cloaca, 22 vent) (Duke, 1984).

*Kidney*

The avian urinary organ consists of a pair of trilobed kidneys and ureters, which transport urine to the urodeum of the cloaca (figure 7). There is no urinary bladder, though the cloaca may serve as its functional equivalent in some species (ostriches).
The structure of avian nephrons is highly heterogeneous, consisting of a variety of reptilian type nephrons and mammalian type nephrons. These mammalian type nephrons have a more complex glomerulus and a loop of Henle. Another feature of the avian kidney is the occurrence of the renal portal system. This system supplies venous blood to the kidneys from the ischiadic and external iliac veins. A valve controls the blood flow towards this portal system (Goldstein and Skadhauge, 2000).

Figure 7. Ventral view of the kidneys of an adult duck (King, 1975).

CONCLUSION

The general pharmacokinetic principles are applicable to bird therapeutics. However, several anatomical and physiological species specific features of birds may cause substantial species differences.
REFERENCES


CHAPTER 1.2

Comparative pharmacokinetics of three NSAIDs in birds

Adapted from:


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ABSTRACT

Information on the pharmacokinetics and pharmacodynamics of anti-inflammatory drugs in birds is scarce. Choice of drug and choice of dosage is usually empirical, since studies of anti-inflammatory drugs are lacking. In this study, three common veterinary non-steroidal anti-inflammatory drugs (NSAIDs) were administered intravenously to five different bird species. Sodium salicylate, flunixin and meloxicam were selected as anti-inflammatory drugs. These NSAIDs were administered intravenously to chickens (*Gallus gallus*), ostriches (*Struthio camelus*), ducks (*Cairina moshata*), turkeys (*Meleagris gallopavo*) and pigeons (*Columba livia*). Plasma concentrations of the drugs were determined by validated high-performance liquid chromatography methods and pharmacokinetic parameters were calculated. Most bird species exhibited a rapid elimination of these drugs. Ostriches had the fastest elimination rate for all three NSAIDs, but there were some interesting species differences. Chickens had a half-life of elimination that was approximately ten times as long as the other bird species for flunixin. The half-life of elimination in chickens and pigeons was three times as long as the other bird species for meloxicam, and for salicylic acid, the half-life of elimination in pigeons was at least three to five times longer than the other bird species.
INTRODUCTION

Salicylic acid is found in some plants and has been used for more than 2000 years in the treatment of various conditions of man and many animal species. Sodium salicylate, a water soluble salt of salicylic acid, may be used in the poultry industry as an anti-inflammatory, antipyretic and analgesic agent. Possible indications are respiratory diseases, digestive coccidial and bacterial infections, inadequate intestinal equilibrium to sustain good weight gain, broiler ascites, heat stress, locomotion disturbances and stimulation of egg production and eggshell quality (Thomas et al., 1966; Proudfoot and Hulan, 1983; Balog and Hester, 1991; Jouglar and Bernard, 1992; McDaniel et al., 1993; Shlosberg et al., 1996; Abd-Ellah et al., 1997; McGeown et al., 1999; Cristòfol et al., 2000). Sodium salicylate is a cheap, water soluble drug and may be of interest to the bird veterinarian. But also newer non-steroidal anti-inflammatory molecules, e.g. flunixin and meloxicam, may offer possibilities for treatment of various conditions in birds. Non steroidal anti-inflammatory drugs act as inhibitors of prostaglandin synthesis (Vane and Botting, 1995). Despite an increasing awareness of animal suffering and the recognition of medical, traumatic and surgical conditions causing pain in animals, there are few pharmacokinetic data of NSAIDs available in birds. Also large species differences in pharmacokinetics of NSAIDs exist (Boothe, 1989). Therefore it is necessary to perform pharmacokinetic studies in the target species to obtain information about the pharmacokinetic behaviour of the drug. Together with the pharmacodynamic information about the drug, a more precise calculation of dosage and dosing interval can be made. The present study provides information on the intravenous disposition of sodium salicylate, flunixin and meloxicam after a single intravenous administration in broiler chickens, ostriches, muscovy ducks, turkeys and pigeons. Also the plasma-protein binding capacity of the three drugs was studied in the five bird species.
MATERIALS AND METHODS

Animals and experimental protocol
The experiments were carried out for each drug in six healthy birds. The broiler chickens (*Gallus gallus*), ostriches (*Struthio camelus*), muscovy ducks (*Cairina moschata*), turkeys (*Meleagris gallopavo*) and pigeons (*Columba livia*) weighed 2.2 ± 0.2 kg, 19 ± 6 kg, 3.0 ± 0.8 kg, 8.0 ± 1.7 kg and 0.45 ± 0.02 kg, respectively. The animals were kept in a group (chickens, ostriches, ducks and turkeys) or individually (pigeons) and fed a commercial bird feed along with tap water *ad libitum*. Sodium salicylate, flunixin and meloxicam were administered by intravenous (IV) bolus injection in the vena basilica (wing vein) at a dose of 25 mg/kg, 1.1 mg/kg and 0.5 mg/kg body weight, respectively. These dosages were selected from pharmacokinetic experiments of these drugs in other animal species. Blood samples were collected in heparinized tubes (Venoject®, Terumo Corp., Tokyo, Japan) from the leg vein (chickens, ducks, turkeys and pigeons) or from the catheterised jugular vein (ostriches) before administration (0) and at different time protocols after the administration of the compound for the different drugs and bird species. For each experiment the total sample volume did not exceed 15% of the blood volume of the animal and optimal sample timing was determined from a preliminary experiment. For example, for pigeons, the smallest bird used, a blood volume of 0.5 ml was taken per sampling point. When taking 10 samples from one pigeon, a total volume of blood of 5 ml is taken. If we assume that pigeons have a blood volume of about 40 ml, the sampling volume would represent 12.5% of the total blood volume. For the larger birds, a sample volume ranging from 1 to 3 ml was taken. Sampling schedules can be derived from the figures 5, 6 and 7. Plasma was separated by centrifugation (2400 x g for 10 min) and the samples were stored at -20 °C until assayed.

Drugs and reagents
A 5% sodium salicylate solution in water, used for intravenous injection, was prepared in the pharmacy of the Faculty of Veterinary Medicine under sterile conditions. Commercially available flunixin (Finadyne®, Schering-Plough n.v., Brussels, Belgium) and meloxicam (Metacam®, Boehringer Ingelheim s.a., Brussels, Belgium) were diluted with sterile NaCl 0.9% and used for intravenous injection. Standards for sodium salicylate, salicylic acid (SA), salicyluric acid (SU), gentisic
Acid (GA) and o-anisic acid (OAA, internal standard (IS) for the SA method) were obtained from Sigma (Bornem, Belgium). Standards of flunixin (FLU), meloxicam (MEL, IS for the FLU method) and piroxicam (PIR, IS for the MEL method) were a gift from the manufacturing companies. The chemical structure of these compounds is given in Figure 1. Solvents of high-performance liquid chromatography (HPLC) grade were obtained from Sigma and Acros (Geel, Belgium) and were used for HPLC analysis.

*Determining flunixin and meloxicam in plasma*

Plasma concentrations of flunixin and meloxicam were determined using a HPLC method with ultraviolet (UV) detection. The samples were analysed on a Thermo Seperations Product (TSP, Fremont, CA, USA) HPLC-system using a P-4000 pump, Model AS 3000 autosampler and a Focus Forward scanning UV-detector. Flunixin was detected at 330 nm and MEL at 355 nm. A 100 x 3 mm I.D. reversed-phase C₁₈ column (5 µm Nucleosil, Chrompack, Antwerpen, Belgium) attached to an appropriate guard column was used. The injection volume was 50 µl. The mobile phase comprised 65% water-acetic acid (99:1, v/v) and 35% acetonitrile. An isocratic elution was performed. The flow rate was 0.7 ml/min.

Samples were prepared by pipetting 0.5 ml of plasma into a 15 ml screw-capped tube, followed by the addition of 50 µl of IS (meloxicam in methanol, 10 µg/ml for the flunixin method; piroxicam in methanol, 10 µg/ml for the meloxicam method), 150 µl of 1 M HCl and 5 ml of diethylether. After centrifugation (2400 rpm, 5 min), the organic layer was transferred to a clean screw-capped tube and evaporated under nitrogen at a temperature of 40°C. The residue was redissolved in 200 µl of the mobile phase, briefly vortexed and 50 µl were injected.

A typical chromatogram of these HPLC-methods is presented in Figure 2 and 3.
Figure 1. Chemical structure of salicylic acid (SA) and its metabolites gentisic acid (GA) and salicyluric acid (SU), flunixin (FLU) and meloxicam (MEL) and the internal standards o-anisic acid (OAA) and piroxicam (PIR).
**Determination of salicylic acid and metabolites in plasma**

Plasma concentrations of salicylic acid and the two major metabolites gentisic acid and salicyluric acid were determined using a HPLC method with UV-detection based on the methods reported by Vree et al. (1994) and Mallikaarjuin et al. (1989). Plasma samples were analysed on a Thermo Seperations Product (TSP, Fremont, CA, USA) HPLC-system using a P-4000 pump, Model AS 3000 autosampler and a Focus Forward scanning UV-detector set at 305 nm. A 250 x 4.6 mm I.D. C$_{18}$ reversed-phase column (5 µm Spherisorb ODS-2, Chrompack) attached to an appropriate guard column was used. The injection volume was 100 µl. The mobile phase comprised 85% water-acetic acid (99:1, v/v) and 15% acetonitrile. A gradient solvent programme was run: 0-4 min: 85/15; 4-20 min: 85/15 – 60/40; 20.1-25 min:85/15. The flow rate was 1 ml/min.

Samples were prepared by pipetting 0.5 ml of plasma into a 15 ml screw-capped tube, followed by the addition of 50 µl of IS (o-anisic acid in methanol, 100 µg/ml), 150 µl of 1 M HCl and 5 ml of diethylether. After centrifugation (2400 rpm, 5 min), the organic layer was transferred to a clean screw-capped tube and evaporated under nitrogen at a temperature of 40°C. The residue was redissolved in 250 µl of the mobile phase, briefly vortexed and 100 µl were injected.

A typical chromatogram of this HPLC-method is presented in figure 4.
Figure 2. HPLC-UV chromatogram of flunixin (RT: 6.948 min) and IS (meloxicam, RT: 9.015 min) after extraction of 1 µg/ml flunixin in turkey plasma.

Figure 3. HPLC-UV chromatogram of meloxicam (RT: 8.662 min) and IS (piroxicam, RT: 4.225 min) after extraction of 1 µg/ml meloxicam in turkey plasma.
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Figure 4. HPLC-UV chromatogram of salicylic acid (RT: 21.053 min) and metabolites gentisic acid (RT: 8.673 min) and salicyluric acid (RT: 12.670 min) and IS (o-anisic acid, RT: 16.100 min) after extraction of 6 µg/ml salicylic acid, gentisic acid and salicyluric acid in chicken plasma.

Validation of the HPLC methods

These methods were validated prior to the start of the analysis. The selectivity of the methods was shown since no interfering peaks from endogenous compounds in the different blank bird plasma were observed with the same retention time as SA, GA, SU, FLU, MEL and their respective IS in the chromatograms of blank samples. Calibration curves were prepared by spiking blank plasma with known concentrations of SA, GA, SU, FLU and MEL. Coefficients of determination were ≥ 0.99 for all components. The limit of detection was determined as three times the signal to noise ratio at the time of elution of the analyte and was: 0.025 µg/ml (FLU), 0.01 µg/ml (MEL), 0.15 µg/ml (SA), 0.1 µg/ml (GA), and 0.1 µg/ml (SU). The limit of quantification was defined as two times the LOD and was: 0.05 µg/ml (FLU), 0.02 µg/ml (MEL), 0.3 µg/ml (SA), 0.2 µg/ml (GA), and 0.2 µg/ml (SU). The accuracy, the within-day precision, and the specificity were checked and all results fell within acceptable limits. These ranges are specified by the European Commission (Anonymous, 2002).
Plasma-protein binding study

Plasma was harvested from blood collected from untreated birds of the different species (chicken, turkey, duck, ostrich and pigeon). Fresh pooled plasma samples were spiked with working solutions of 1, 10 and 20 µg/ml flunixine, 0.5, 5 and 10 µg/ml meloxicam and 5, 25 and 50 µg/ml salicylic acid. These concentrations were chosen on the basis of the obtained plasma concentrations in the pharmacokinetic studies. Three samples of each concentration were added to a commercial ultrafiltration (UF) device (Microcon® YM-10, Amicon bioseparations, Millipore corporation, Bedford, USA). The UF devices were centrifuged at a fixed (28°) angle (Ultracentrifuge, Abbott Laboratories, Ludwigshafen, Germany) at 1200 g for 1 h. Calculation of the percentage of the drug bound to plasma proteins was done by the following equation:

\[
\text{Bound drug} = \frac{(\text{Initial plasma concentration} - \text{UF concentration})}{\text{Initial plasma concentration}} \times 100
\]

Analysis of data

Analyses were performed for each data set independently. The pharmacokinetic parameters (elimination rate constant: \(k_{el}\), half-life of elimination: \(t_{1/2el}\), volume of distribution: \(V_{d(area)}\), area under the curve from 0 to infinity: AUC, total clearance: Cl, mean residence time: MRT) were calculated with a computer program (MW/PHARM version 3.15, Groningen, The Netherlands). Pharmacokinetic analysis was performed using a least-squares non-linear regression analysis programme. The pharmacokinetic parameters were determined with open compartment models. The best fitting model was selected after a curve stripping procedure based on curve appearance and the coefficient of determination of the sum of squares. The following equations were used to describe the concentration-time curves of the three NSAIDs after intravenous administration: 

\[C = C(0)e^{kt}\] (a one compartment model); 

\[C = A_1e^{\alpha t} + A_2e^{\beta t}\] (a two compartment model), where \(C\) is the plasma concentration, \(C(0)\) is the extrapolated initial concentration, \(k\) is the elimination rate constant, \(A_1\) and \(A_2\) are mathematical coefficients, \(\alpha\) is the distribution rate constant, \(\beta\) is the elimination rate constant and \(t\) is the time. The different pharmacokinetic parameters were compared between the species using analysis of variance. Bird species were pairwise compared...
using the Tukey's multiple comparisons technique with a family confidence coefficient equal to 95%.

RESULTS

Pharmacokinetic study
The mean plasma concentrations of salicylic acid, flunixin and meloxicam in each bird species plotted on a semi-logarithmic scale are presented in figures 5, 6 and 7, respectively. In the analytical method of salicylic acid, two metabolites (salicyluric acid and gentisic acid) could be quantitated in the same run. In none of the bird species SU could be found in the plasma of the salicylate treated birds. Only in broiler chickens, GA was detected at concentrations of maximum 0.8 µg/ml. In all of the bird species SA was best described as a one compartment open model. The data obtained for FLU and MEL were best described as two compartment open models for chickens and ostriches, and as one compartment open models for ducks, pigeons and turkeys. In the meloxicam experiment for ducks and turkeys and the flunixin experiment for turkeys the curve of two animals was best fitted to a two compartment open model. In table 1, the mean pharmacokinetic parameters of SA, FLU and MEL after intravenous administration of 25 mg/kg of SA, 1.1 mg/kg FLU and 0.5 mg/kg MEL in the five different bird species are shown.
Figure 5. Mean plasma concentrations (n=6) of flunixin in five bird species (ostriches, turkeys, ducks, chickens, pigeons) after an intravenous bolus administration of 1.1 mg/kg FLU.

Figure 6. Mean plasma concentrations (n=6) of meloxicam in five bird species (ostriches, turkeys, ducks, chickens, pigeons) after an intravenous bolus administration of 0.5 mg/kg MEL.
Figure 7. Mean plasma concentrations (n=6) of salicylic acid in five bird species (ostriches, turkeys, ducks, chickens, pigeons) after an intravenous bolus administration of 25 mg/kg sodium salicylate.
Table 1. Pharmacokinetic parameters of salicylic acid (SA), flunixin (FLU) and meloxicam (MEL) in plasma after intravenous administration of 25 mg/kg of SA, 1.1 mg/kg FLU and 0.5 mg/kg MEL (n = 6, mean ± sd) in 5 different bird species. The pharmacokinetic parameters are: AUC: area under the curve from 0 to infinity, Cl: total clearance, Vd(area): volume of distribution, t1/2el: half-life of elimination, k_el: elimination constant, MRT: mean residence time. Species with different letters are significantly different at a family confidence coefficient = 95%.

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<td>MRT (h)</td>
<td>3.89^{a}</td>
<td>1.49</td>
<td>0.77^{bc}</td>
<td>0.20</td>
<td>1.47^{b}</td>
<td>0.27</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylic acid</td>
<td>373.70^{a}</td>
<td>73.60</td>
<td>150.50^{b}</td>
<td>59.60</td>
<td>55.29^{c}</td>
<td>9.29</td>
</tr>
<tr>
<td>Cl (l/h*kg)</td>
<td>0.069^{a}</td>
<td>0.013</td>
<td>0.160^{ac}</td>
<td>0.090</td>
<td>0.46^{b}</td>
<td>0.08</td>
</tr>
<tr>
<td>Vd(area) (l/kg)</td>
<td>1.48^{a}</td>
<td>0.13</td>
<td>1.580^{a}</td>
<td>0.250</td>
<td>2.05^{b}</td>
<td>0.38</td>
</tr>
<tr>
<td>t_{1/2el} (h)</td>
<td>15.28^{a}</td>
<td>2.56</td>
<td>6.86^{b}</td>
<td>3.56</td>
<td>3.13^{c}</td>
<td>0.78</td>
</tr>
<tr>
<td>k_el (h^{-1})</td>
<td>0.046^{a}</td>
<td>0.008</td>
<td>0.13^{ab}</td>
<td>0.07</td>
<td>0.23^{b}</td>
<td>0.05</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>22.05^{a}</td>
<td>3.70</td>
<td>9.90^{b}</td>
<td>5.13</td>
<td>4.52^{c}</td>
<td>1.13</td>
</tr>
</tbody>
</table>
Plasma-protein binding study

The mean and the standard deviations (n=3) of the calculated percentage of binding of FLU, MEL and SA to plasma proteins of ostriches, ducks, chickens, turkeys and pigeons is presented in figures 8, 9 and 10.

Figure 8. Plasma-protein binding capacity (± SD) of ostrich, duck, chicken, turkey and pigeon plasma for flunixin at a concentration of 1, 10 and 20 µg/ml.
Figure 9. Plasma-protein binding capacity (± SD) of ostrich, duck, chicken, turkey and pigeon plasma for meloxicam at a concentration of 0.5, 5 and 10 µg/ml.

Figure 10. Plasma-protein binding capacity (± SD) of ostrich, duck, chicken, turkey and pigeon plasma for salicylic acid at a concentration of 5, 25 and 50 µg/ml.
DISCUSSION

Comparative mean pharmacokinetic parameters of the three drugs in different mammals are summarised in table 2. In this table, standard deviations of the parameters are not given, but some variability is to be expected due to individual variability and difference in calculation manner. The values for volume of distribution and clearance were related to the body weight for better comparison between species.

Table 2. Mean value of major pharmacokinetic parameters of salicylic acid, flunixin and meloxicam in mammals.

<table>
<thead>
<tr>
<th></th>
<th>Vd(area) (l/kg)</th>
<th>Cl (l/h*kg)</th>
<th>t₁/₂ el (h)</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>Rabbits</td>
<td>0.249</td>
<td>0.043</td>
<td>4.29</td>
</tr>
<tr>
<td></td>
<td>Dogs</td>
<td>0.285</td>
<td>0.041</td>
<td>4.49</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>0.176</td>
<td>0.253</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>Goats</td>
<td>0.138</td>
<td>0.193</td>
<td>0.50</td>
</tr>
<tr>
<td>FLU</td>
<td>Camels</td>
<td>0.489</td>
<td>0.089</td>
<td>3.76</td>
</tr>
<tr>
<td></td>
<td>Horses</td>
<td>0.317</td>
<td>0.058</td>
<td>3.37</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>0.166</td>
<td>0.060</td>
<td>3.82</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>0.782</td>
<td>0.115</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>Dogs</td>
<td>0.348</td>
<td>0.064</td>
<td>3.75</td>
</tr>
<tr>
<td>MEL</td>
<td>Horses</td>
<td>0.160</td>
<td>0.042</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Humans</td>
<td>0.190</td>
<td>0.011</td>
<td>13.7</td>
</tr>
</tbody>
</table>

The volume of distribution of salicylic acid was very large in most bird species, only ostriches have a low volume, comparable to that of rabbits and dogs (Short et al., 1990b; Waters et al., 1993). The clearance of SA was fairly rapid, comparable to that of cattle and goats (Short et al., 1990a), for most bird species, except for pigeons. Consequently, the biological half-life of SA was longer in pigeons than in the other
bird species. Metabolism of salicylic acid can occur through conjugation with glycine
to form salicyluric acid (SU), oxidation to 2,5-dihydroxybenzoic acid (gentisic acid,
GA), glucuronide formation and the formation of some minor metabolites. In many
mammalian species (man, horse, cattle, goat, rat), salicyluric acid is the major
metabolite. In rabbits it is a minor metabolite and the only metabolite detectable in
plasma (Short et al., 1990b). In our experiments in birds, however, only GA could be
detected in the chicken plasma at low concentrations. No SU was found in the plasma.
This could mean that rabbits and birds are deficient in their ability to conjugate SA
with glycine, relative to other species. No other metabolites were found in the plasma,
but this analytical method did not detect other amino acid conjugates. Differences in
amino acid conjugation in birds and fish are described for benzoic acid (Pan and
Fouts, 1978, Burke et al., 1987). Also the method did not detect the glucuronide
conjugates, since there was no glucuronidase treatment during the sample preparation.
Chickens, however, have a low level of liver glucuronyl transferase (Denbow, 2000)
and in other species glucuronide formation seems to be only a minor pathway. In
goats and cattle, no glucuronide conjugates were found, in humans glucuronide
formation is around 10% of the excreted dose, in horses it is only 2%. For humans,
there are reports that formation seems to be highly variable, ranging from 0.8-40% of
the excreted dose (Short et al., 1990a).

The volume of distribution of flunixin was generally lower in birds than in mammals
(Wasfi et al., 1998; Odensvik, 1995; Landoni and Lees, 1995, Welsh et al., 1993;
Hardie et al., 1985). The clearance of flunixin in birds was more variable than in
mammals and was generally faster. As a result, the biological half-life of flunixin was
generally faster in birds, except for chickens. Ostriches have a very short half-life of
about 10 minutes. Metabolism of flunixin can occur through oxidation and
glucuronidation of either the parent molecule or the oxidised flunixin. In camels and
dogs glucuronidation of the parent molecule seems to be the major pathway (Wasfi et
al., 1998; Brady et al., 1998). In horses a hydroxy metabolite is formed and
conjugation reactions have been suggested (Jaussaud et al., 1987).

The volume of distribution of meloxicam was lower in chickens, turkeys and ducks,
of the same magnitude in pigeons and much larger in ostriches than in horses and
humans (Lees et al., 1991; Schmid et al., 1995). All the bird species, except
ostriches, cleared meloxicam as slowly as horses and humans. This results in a variable half-life of elimination for these bird species. Ostriches had the fastest half-life. Other data on half-lives of meloxicam in different species also show a large variation between species: cattle (13 h), rat (11 h), dog (12-36 h), human (20-50 h), mini-pig (4 h) (Lees et al., 1991). Metabolism of meloxicam can occur through oxidation of the parent molecule and further oxidising the metabolites. Meloxicam is eliminated partly in urine and partly in faeces of humans and rats. Only traces of the parent drug can be recovered from excreta (Schmid et al., 1995).

Excretion studies of urine and faeces could further elucidate the metabolism and excretion pattern of salicylic acid, flunixin and meloxicam in birds. Also, the formation of metabolites can be investigated with more advanced analytical methods and use of β-glucuronidase in the sample preparation.

The plasma protein binding of flunixin and meloxicam follows a similar pattern in the five bird species. The protein binding capacity of the plasma was between 65 and 80% for both drugs at the two low concentrations in the different species. The protein binding of flunixin and meloxicam at the high concentration was somewhat lower for all species. This may suggest a concentration dependent saturation of the binding sites of the plasma proteins for flunixin and meloxicam. The plasma protein binding of salicylic acid was more variable across species. No dose related difference was seen with salicylic acid. Especially the protein binding of salicylic acid in pigeon plasma was striking. A low protein binding can cause a high volume of distribution since the free fraction of the drug in the blood is high and available for distribution. This is true for pigeons, since they have a relatively high volume of distribution. However, other factors must play a role in the distribution process, since birds with a moderate protein binding (e.g. turkeys) also have a high volume of distribution.

The bird data were also analysed to determine any correlation to body mass. In figures 11, 12 and 13, the allometric analysis of the log of the half-life versus the log of the body weight of the birds of FLU, MEL and SA are shown. The log of the values for elimination half-life, clearance and volume of distribution was related to the log of the body mass using the allometric equation: \( Y = aW^b \). The function \( Y \) represents the parameter of concern (half-life of elimination, clearance or volume of distribution), a
is a coefficient typical of every drug (intercept), $W$ is the species average body weight, and $b$ is the scaling exponent (Riviere et al., 1997). In none of the drugs and none of the parameters a good correlation could be shown. Only for the half-life of SA, a reasonably good correlation was seen. However, in all three of the drugs the half-life, considered as the most robust parameter for interspecies scaling, showed a negative correlation with the body weight. This is mainly because the largest birds (the ostriches) have the fastest elimination half-life and this contradicts an allometric relationship with weight. The opposite is true for SA in pigeons, the smallest bird, which have the longest half-life. Reasons for these findings are not known, but changes between species in elimination and protein binding are a possible explanation. Drugs that are eliminated mainly by renal filtration processes are expected more to correlate allometrically with weight across species, since glomerular filtration is a reflection of cardiac output and basal metabolic rate. Thus, drugs with a poor allometric analysis may be eliminated by a 'capacity limited' biotransformation, and this is dependent upon the intrinsic ability of that species and individual to metabolise the drug. Differences in protein binding across species would be expected to affect clearance, volume of distribution and the fraction of a dose that is able to interact with receptors (Riviere et al., 1997). Also, only five bird species were considered in the allometric analysis of these drugs and the log of the body weight ratio was fairly small (1.7). Consequently, the results for these drugs should be taken with caution (Riviere et al., 1997). However, for these drugs, allometry does not seem to be a valuable tool for extrapolating doses between bird species. This implicates that the pharmacokinetics of every NSAID need to be tested in the target bird species.
CHAPTER 1.2

$$y = 1.1069x^{-0.4043}$$
$$R^2 = 0.2131$$

Figure 11. Allometric plot obtained for flunixin based on five bird species.

$$y = 2.0563x^{-0.4157}$$
$$R^2 = 0.5948$$

Figure 12. Allometric plot obtained for meloxicam based on five bird species.
All NSAIDs induce undesirable side effects. Most adverse reactions reflect the inhibitory effects on prostaglandin activity with manifestations most often being gastrointestinal in nature. Salicylates cause local damage due to ‘backdiffusion’ of acid, which causes injury to mucosal cells and submucosal capillaries (Boothe, 1989). Other side effects can occur in the hematopoetic or the renal system. In these studies, no clinical side effects were noticed. No post mortem examinations were carried out. Further research could characterise the effects of multiple administrations or continuous administration in feed or drinking water.

Based on the pharmacokinetic parameters of SA, FLU and MEL after intravenous administration, one may conclude that these NSAIDs are eliminated rapidly in most bird species, especially in ostriches. They may offer possibilities for treatment of various conditions in birds, but further research on pharmacokinetics (other routes of administration) and pharmacodynamics of NSAIDs in birds is required. This information should be the starting point of further research to the pharmacokinetics, metabolic profile, pharmacodynamics and side effects of non-steroidal anti-inflammatory drugs in birds.
ACKNOWLEDGEMENTS

The valuable cooperation of Prof. Dr. L. Duchateau in the statistical analysis and the technical assistance of Miss H. Lippens in the animal experiments is gratefully acknowledged.
REFERENCES


Biotransformation of salicylic acid in chickens and pigeons

Adapted from:
(2003) Comparative metabolism of sodium salicylate in broiler chickens and homing pigeons

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*Laboratory for Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium
ABSTRACT

Information on the metabolism of anti-inflammatory drugs in birds is scarce. Excretion studies and chromatographic analysis of anti-inflammatory drugs and their metabolites are lacking. In this study, sodium salicylate was administered intravenously to broiler chickens and pigeons. The combined urine and faeces droppings were collected at different time intervals after administration. The concentrations of salicylic acid, gentisic acid, the amino acid conjugation product of salicylic acid with glycine and the amino acid conjugation product of salicylic acid with ornithine were determined in the excreta by validated high-performance liquid chromatography methods with mass spectrometric detection. A marked species difference was seen. Chickens conjugated salicylic acid with ornithine, while pigeons conjugated salicylic acid with glycine. This may be a possible explanation for the difference in the plasma half-life of salicylic acid between chickens and pigeons.
INTRODUCTION

In comparison to mammals, the knowledge of xenobiotic metabolism in birds is very limited, especially with regard to drugs containing carboxylic acid, such as salicylates. In the body, a drug can be metabolised in two phases. In the first phase (phase I), oxidations, reductions and hydrolyses take place. The resulting metabolites may subsequently undergo conjugation reactions in the second phase (phase II). A wide range of carboxylic acids in drugs, herbicides and pesticides can undergo conjugation reactions in birds and mammals. The fate of the simplest aromatic carboxylic acid, benzoic acid, has been studied extensively as a model compound for biotransformation reactions in animals, where it can undergo conjugation with a sugar (glucuronic acid in vertebrates, glucose in insects) and/or an amino acid (Burke et al., 1987). The nature of the amino acid involved depends both on the animal species as on the chemical structure of the carboxylic acid. In general, the amino acids participating in conjugation reactions are glycine and to a lesser extent glutamine, taurine and ornithine. Glycine is most commonly encountered and is utilised by a wide range of species, including humans, for the conjugation of carboxylic acids. Glutamine conjugation is restricted to arylacetic acids in man and other primate species. Taurine conjugation is also relatively widespread in its species occurrence (particularly in carnivores), but is restricted in terms of acids undergoing this conjugation to arylacetic and cholic acid derivatives (Kasuya et al., 1999). There have only been a few reports on ornithine conjugation and the authors indicated that ornithine conjugation is restricted in the species occurrence. It has been found that ornithine conjugation occurs only in some birds and reptilian species but has not been reported in mammalian species (Igarashi et al., 1992). The major metabolite of benzoic acid in most mammals and in birds belonging to the order Columbiformes (pigeon, woodpigeon, dove) is hippurate (benzoylglycine), whereas in Galliformes (chicken, turkey, quail) and Anseriformes (duck, goose) it is ornithurate (α,δ-dibenzoylornithine) (Williams, 1967; Pan and Fouts, 1978; Seymour et al., 1988).

The use of a non-steroidal anti-inflammatory drug in bird medicine has been controversial. In poultry industry, no improvements in economical parameters such as growth and egg production indications are seen after the use of NSAIDs. Therefore, to date no commercial formulations are available for use in birds in Belgium.
Nevertheless, valid indications for the use of an NSAID in birds exist (Bauck, 1990). The pharmacokinetics of sodium salicylate, a simple carboxylic acid drug, in several bird species have been described in chapter 1.2. A marked species difference in pharmacokinetic parameters for this drug was found between chickens and pigeons. The elimination half-life of sodium salicylate in pigeons was five times longer than in chickens. A reason for this difference in half-life may be the species difference in biotransformation of the drug, as is described for benzoic acid. In this study, the excretion pattern of salicylic acid and its metabolites in both chicken and pigeon excreta was investigated quantitatively with a liquid chromatographic method with mass spectrometry.

MATERIALS AND METHODS

Animals and study design
The experiments were carried out on six healthy broiler chickens and six pigeons, weighing 1.8 ± 0.2 kg, and 0.45 ± 0.02 kg, respectively. The animals were kept in group (chickens) or individually (pigeons) and fed a commercial bird feed along with tap water ad libitum. Sodium salicylate was administered by intravenous bolus injection in the vena basilica (wing vein) at a dose of 25 mg/kg body weight. This dosage was selected from pharmacokinetic experiments of this drug in these bird species. Combined urine and faeces samples were collected and pooled from the different birds at several time points after the administration of sodium salicylate. The sampling schedule for excreta of chickens was as follows: 0, 2, 4, 6, 8, 12, 24 and 36 h and for pigeons: 0, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h. The collected excreta were diluted with water (1/1, w/w) and mixed using a rod blender. Thereafter, a further homogenisation was performed using a mechanical stirring instrument (CAT R50, 30-1600 rpm). The samples were stored at -20 °C until assayed.

Reference compounds
The following compounds were obtained from Sigma (Steinheim, Germany): sodium salicylate, salicylic acid (SA), gentisic acid (GA): an oxidation product of salicylate, salicyluric acid (SU): the glycine conjugate of salicylic acid and the internal standard, phenoxy acetic acid (PAA). The double conjugated ornithine metabolite of salicylic
acid (SO) was not commercially available and was synthesised as described by Hillaert et al. (2003). The structure was assigned by nuclear magnetic resonance ($^1$H-NMR and $^{13}$C-NMR) and mass spectrometry (MS). Figure 1 shows the chemical structure of the investigated compounds.

Sample preparation
One gram of combined urine and faeces homogenate was transferred in a capped 5 ml tube and spiked with 100 µl of the working solution of 100 µg/ml of the internal standard phenoxy acetic acid. A volume of 5 ml of water was added. The tube was vortexed during 30 s and centrifuged at 2500 rpm for 10 min. One ml of the supernatant was diluted to 50.0 ml in a volumetric flask. A 10 µl aliquot of the aqueous phase was injected into the LC-MS/MS instrument.

Liquid chromatography and mass spectrometry
The HPLC system consisted of an Alliance Type 2690 separation pump with a column heater module and an autosampler cooling device (all from Waters, Milford, USA). Chromatographic separation was achieved using a reversed-phase Nucleosil C$_{18}$ column (125 x 3 mm ID, particle size 5 µm), in combination with a guard column of the same type (8 x 3 mm ID), from Macherey-Nagel GmbH & Co. (Düren, Germany). The mobile phase A was a solution of 1% acetic acid in water (v/v), while the mobile phase B was methanol. The flow rate was 0.2 ml/min for a total run time of 20 min. A solvent gradient elution was performed according to following scheme: 0-1 min: 60% A and 40% B ; 1-9 min: linear change to 30% A and 70% B ; 9-11 min: 30% A and 70% B ; 11-20 min: fast change to 60% A and 40% B.
Figure 1. Chemical structure of salicylic acid (SA) and its metabolites gentisic acid (GA), salicyluric acid (SU) and the double conjugated ornithine metabolite (SO) and the internal standard phenoxy acetic acid (PAA).

The HPLC column effluent was pumped to a Quattro Ultima triple quadrupole mass spectrometer instrument (Micromass, Manchester, UK), equipped with an ESI (electrospray ionization) z-spray ion source, which was operated in the negative ion MS/MS mode. The instrument was first calibrated with a 1 mg/ml solution of sodium iodide (NaI) in the 100-1550 m/z range. Thereafter, the instrument was tuned by direct infusion of a solution of 1 µg/ml of all the components in the ESI source. For quantitative purposes, the instrument was operated in the multiple reaction monitoring
(MRM) mode. A chromatogram of these drugs in chicken and pigeon excreta is shown in figure 2 and 3.

Figure 2. LC/ESI-MS/MS ion chromatogram of IS (trace A), SO (trace B), GA (trace C) and SA (trace D) fortified at 50 µg/g in chicken excreta.
Figure 3. LC/ESI-MS/MS ion chromatogram of IS (trace A), SU (trace B), GA (trace C) and SA (trace D) fortified at 50 µg/g in pigeon excreta.

Validation of the methods
The proposed method for the quantitative determination of salicylic acid and metabolites in excreta was validated by a set of parameters which are in compliance with the recommendations as defined by the European Commission (Anonymous, 2002, Heitzman, 1994). The validation parameters examined were the linearity (between 0 and 250 µg/g in fortified excreta), the accuracy, the within-day precision, the limit of detection (LOD), the limit of quantification (LOQ) and the specificity. All
results fell within acceptable limits. The LOQ of the method was 5 µg/g for all compounds in both chicken and pigeon excreta. Using a signal-to-noise ratio of 3:1, the LOD was 2.9, 2.1 and 0.9 µg/g for SA, GA and SU in pigeon excreta, and 1.7, 0.7 and 3.2 µg/g for SA, SO and GA in chicken excreta, respectively.

RESULTS

The average concentration of the drug and metabolites in chicken- and pigeon excreta are shown in figure 4 and 5. In the chicken excreta SA, GA and SO were found, while in pigeon excreta SA, GA and SU were found. In the chicken excreta, SA was detected up to 24 h after administration. For the metabolites, GA and SO were quantified and reached their maximum concentration about 2 h later than SA. In pigeon excreta, SA was measured up to 48 h after treatment and remarkably, no SO was detected. The SU conjugate and also GA were seen in the excreta, and SU could still be quantified at 72 h post-treatment.

DISCUSSION

By comparison with mammals, the knowledge of xenobiotic metabolism in birds is very limited. Moreover, there are nearly 9000 living bird species and only 13 of these have been tested with one or more organic acids for amino acid conjugation (Huckle et al., 1982). According to these studies, the Galliformes form mainly ornithine conjugates with benzoic and 4-aminobenzoic acids and ornithine and taurine conjugates with arylacetic acids. Anseriformes also form ornithine conjugates, but have not been tested with arylacetic acids. By contrast, Columbiformes form glycine conjugates with benzoic acid, and glycine and/or taurine conjugates with arylacetic acids. In the one investigation into the conjugation of benzoic acids in Passeriformes (which comprise about 5000 of the 9000 known species of living birds) and Psitacciformes, no amino acid conjugates were detected (Huckle et al., 1982).
The pharmacokinetic parameters after intravenous administration of sodium salicylate at a dose of 25 mg/kg in chickens and pigeons have been described in chapter 1.2. The plasma elimination half-life of salicylic acid in these two bird species is markedly different. The average elimination half-life in pigeons (15.28 h) is almost 5 times longer than the elimination half-life in chickens (3.18 h). This finding can have several possible causes. The volume of distribution of salicylic acid is larger in pigeons (1.48 l/kg) than in chickens (0.95 l/kg). This can be caused by a lower degree of plasma protein binding in pigeons. The degree of plasma protein binding for salicylic acid in chicken and pigeon plasma was studied in chapter 1.2. and was about 62% and 15%, respectively. Also, the clearance of salicylic acid is larger in chickens (0.21 l/h.kg) than in pigeons (0.069 l/h.kg). This means that a smaller volume is cleared from salicylic acid in pigeons than in chickens. The level and the speed of biotransformation of salicylic acid may play a significant role in this phenomenon.
Figure 5. Mean concentrations (n=6) of salicylic acid (SA), gentisic acid (GA) and the glycine conjugate of salicylic acid (SU) in the excreta of pigeons after an intravenous bolus administration of 25 mg/kg sodium salicylate.

Metabolism of salicylic acid can occur through oxidation to 2,5-dihydroxybenzoic acid (gentisic acid, GA), conjugation with glycine to form salicyluric acid (SU), conjugation with ornithine to form a double conjugated ornithine metabolite (SO), glucuronide formation and the formation of some minor metabolites. In many mammalian species (man, horse, cattle, goat, rat), salicyluric acid is the major metabolite. In rabbits it is a minor metabolite and the only metabolite detectable in plasma (Short et al., 1990b). In our previous pharmacokinetic experiments in chickens and pigeons, however, only GA could be detected in the chicken plasma at low concentrations. No SU was found in the plasma. These samples were analysed with an HPLC-UV detection method. Preliminary investigations of the same plasma samples with an improved LC-MS/MS method revealed also the presence of SO in chicken plasma from 30 min to 12 h after administration. However, in pigeon plasma no metabolites could be found with UV detection, nor with MS detection. Furthermore, the salicyluric acid metabolite was only detected in the pigeon excreta, even at a high concentration. This could mean that formation of that metabolite occurs mainly in the
kidney and is rapidly excreted through the urine without appearing at detectable concentrations in the pigeon plasma. Therefore, an additional experiment was performed to investigate the presence of SA and metabolites in kidney and/or liver samples from pigeons. In two treated pigeons the liver and kidneys were collected 28 h after intravenous administration of 25 mg/kg sodium salicylate. Analysis of these samples with the MS/MS method revealed the presence of salicylic acid in pigeon liver and salicylic acid and salicyluric acid in pigeon kidney. In other species, the kidney also seems to be an important site for glycine conjugation but the relative importance of the liver and the kidney for glycine conjugation varies with the species and substrates. In man, 68% of the glycine conjugation of salicylic acid was renal and 32% hepatic. In rhesus monkeys and in vitro in the mouse, the conversion of salicylic acid to salicyluric acid occurs exclusively in the kidney (Kasuya et al., 1999). Our experiments suggest that the pigeon kidney is also the main site for glycine conjugation of salicylic acid in pigeon. The site of ornithine conjugation in chickens was also investigated in an additional experiment. In two treated chickens the liver and kidneys were collected 3 h after intravenous administration of 25 mg/kg sodium salicylate. Analysis of these samples with the MS/MS method revealed the presence of salicylic acid, gentisic acid and the ornithine conjugate, both in chicken liver and kidney. However, the presence of SO in the kidney could also be attributed to excretion. Thus, our experiments indicate that both organs may be important for the ornithine conjugation in chickens. The excreta of chickens and pigeons were also scanned in our experiments for the occurrence of a possible taurine conjugate of salicylic acid, but no precursor ion nor product ions, based on the supposed molecular formula, were found.

Further research to the biotransformation of salicylic acid in birds can be the study of the formation of glucuronides, since the method did not detect the glucuronide conjugates. Therefore, a glucuronidase treatment should be included during the sample preparation. Chickens have a low level of liver glucuronyl transferase (Denbow, 2000) and in other species glucuronide formation seems to be only a minor pathway (Short et al., 1990a). Furthermore, in several mammal species the rate of glycine conjugation is greater than the rate of glucuronidation, which occurs almost exclusively in the liver (Seymour et al., 1987a). In goats and cattle, no glucuronide conjugates were found, in horses it is only 2%, in humans glucuronide formation is
around 10% of the excreted dose. For humans, there are reports that formation seems to be highly variable, ranging from 0.8-40% of the excreted dose (Short et al., 1990a). These results confirm the findings of other authors that in general chickens form ornithine conjugates with simple carboxylic acid drugs, whereas pigeons form glycine conjugates (Williams, 1967; Idle et al., 1976; Seymour et al., 1987b). Elimination of the parent compound and some oxidation seems to occur in both species. Current information shows a slower elimination pattern for salicylic acid in pigeons and a long excretion time for salicyluric acid in pigeon droppings. Based on preliminary information, the main site of glycine conjugation in the pigeon seems to be the kidney and for ornithine conjugation in the chicken, both the liver and the kidney seem to be important.

ACKNOWLEDGEMENTS

The authors thank Hilde Lippens for her excellent technical assistance.
REFERENCES


Pharmacodynamics of NSAIDs in birds

‘Pharmacodynamics is what the drug does to the body’
The inflammatory response in birds

Translated and adapted from:

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ABSTRACT

Inflammation is a complex mechanism designed to protect the organism against different stimuli. It has been and still is being extensively studied in mammals. Literature on inflammation and anti-inflammatory drugs in birds is limited. In this article the current knowledge is reviewed about inflammation in birds and the models used to study it. A comparison with inflammation in mammals is made. More attention is given to the acute phase of inflammation. Different aspects of inflammation are highlighted: increased vascular permeability, leukocyte changes, acute phase proteins and inflammation mediators.
INTRODUCTION

Inflammation is a complex homeostatic process designed to protect animals against trauma and infection caused by chemical, biological and physical stimuli. Inflammation serves to destroy, dilute, or wall off the injurious agent, and it sets into motion a series of events that, as far as possible, heal and reconstitute the damaged tissue. If the inflammatory stimulus is neutralised, the injured tissue is replaced by regeneration of native parenchymal cells, by filling of the defect with fibroblastic scar tissue (scarring), or most commonly by a combination of these two processes (Cotran et al., 1989; Lees et al., 1991). The inflammatory response is usually classified into acute and chronic phases. The cardinal signs of the acute phase were already described in the 1st century AD as calor, rubor, tumor and dolor by Celsus. Later, in the 19th century functio laesa was added by Virchow (figure 1). These changes are attributable to events involving the microvasculature and blood cells. Characteristics of acute inflammation are discussed further.

Figure 1. The cardinal signs of acute inflammation (Forscher, 1968).
Chronic inflammation develops if the irritant stimulus persists and the acute inflammatory process fails to resolve. Macrophages form a syncytium around the area of immunogen and together with fibroblasts and fibrin wall off the site forming a granuloma. These are not associated with the cardinal signs that characterise acute inflammation. Following signs may be observed microscopically: cell death or tissue necrosis, scar tissue, inflammatory cell infiltrates that predominantly consist of lymphocytes, plasma cells, macrophages and multi-nucleated giant cells. These inflammatory processes tend to develop much faster in birds than in mammals, rapidly leading to a chronic lesion (Klasing, 1991; Frédette and Boulianne, 1994). In mammals, extensive investigations have already been made and disease-specific models are being developed. Avian inflammation has only recently received greater attention.

INFLAMMATION MODELS IN BIRDS

Inflammation has been studied over the years with different models and methods. Usually the inflammation is evoked by simple injection of an irritating substance or a bacteriological toxin. Other models insert a foreign body either subcutaneously or intraperitoneally in the chicken. From these foreign bodies, inflammatory exudate or emigrating cells can be extracted and investigated (Higgins, 1984; Vegad and Katiyar, 1995). Inflammation can be initiated by different stimuli: mechanical, thermal, infectious, chemical and immunological. Katiyar et al. (1992) induced inflammation with punch wounds in the chicken skin. He also reported the inflammatory reaction following a thermal injury in the chicken skin. In the early seventies, Carlson (1972) and Nair (1973) injected Staphylococcus aureus suspended in sterile normal saline into wingwebs, breast muscles and subcutaneously in chickens. Nair (1973) also studied the inflammatory reaction to Ascaris suum and Toxocara canis parasites. Several authors already used chemical injury to study inflammation in the chicken. Turpentine, normal saline, carrageenan, endotoxin, phytohaemagglutinin, concavalin A, dextran sulphate, trypan blue, freund’s complete adjuvant, urate crystals, fibrinogen, talcum, xylol and dinitrochlorobenzene were injected intradermally, subcutaneously, intramuscularly, intraperitoneally or intrarticularly (Vegad and Katiyar, 1995). Also anaphylactic reactions (passive cutaneous
anaphylaxis, passive mesenteric anaphylaxis), arthus reactions and delayed hypersensitivity reaction have been studied in the chicken (Vegad and Katiyar, 1995). The different facets of inflammation can be investigated with different methods. Pathological methods can be used to study the ‘macroscopical’ cardinal signs of inflammation: calor (fever, skin temperature), tumor (swelling), rubor (redness), dolor (pain), functio laesa (impaired function). Histological methods study the ‘microscopical’ changes that are induced by an inflammation: either on cellular level (counting different number of leukocytes) or an assessment of increased vascular permeability (dye technique and colloidal carbon technique). Other laboratory methods are needed to investigate the mediation of the inflammatory process: quantitation of mediators and acute phase proteins, in vitro methods with leukocytes.

INCREASED VASCULAR PERMEABILITY

The initial vascular response is a transient constriction of arterioles, which is rapidly followed by arteriolar vasodilatation and increased blood flow leading to heat and redness. The permeability of the small postcapillary venules is increased, thus enhancing the passage of plasma proteins (especially albumin) from the circulation into surrounding tissues. When extravasation of plasma exceeds the capacity of the lymphatic drainage system to remove fluid, oedematous swelling is observed. These microcirculatory changes are caused by the release and actions of chemical mediators, including histamine, serotonin, leukotrienes, prostaglandins and others. The actions of these mediators of permeability are mainly restricted to venules and small veins only (Ito and Böhm, 1986; Vegad and Katiyar, 1995). These actions are immediate and either monophasic or biphasic. An intradermal injection of xylol, carrageenan, E. coli endotoxin and punch wounding of the skin showed a monophasic permeability response. Intraperitoneal or intradermal injection of turpentine and passive cutaneous anaphylaxis revealed a biphasic pattern. This comprised an immediate reaction and a delayed, more prolonged response. These studies suggest that the patterns of permeability response in the acute inflammatory reaction in the chicken are both biphasic and monophasic, which is similar to those occurring in mammals (Vegad and Katiyar, 1995). The pain associated with acute inflammation is caused by mediators such as histamine and bradykinin that stimulate nociceptors and prostaglandins.
(PGE$_2$, PGI$_2$) that sensitise the sensory nerve endings to the actions of these mediators.

LEUKOCYTES

Leukocyte changes in chicken blood after an intramuscular turpentine injection were described by Latimer et al. (1988). They found significant heterophilia and leukocytosis, which peaked at 12 hours and 3 days, respectively. Mean monocyte and lymphocyte counts peaked at 2 and 3 days, respectively. Basophil and eosinophil counts were erratic. Toxic changes of heterophils were most apparent during intense left shifts (at 12 and 24 hours) and consisted of swelling, degranulation, cytoplasmic vacuolisation and cytoplasmic basophilia. The turpentine model of leukocytosis with a primary heterophilia is consistent with the haematological response to acute staphylococcal tenosynovitis and coccidiosis in chickens (Harmon, 1998).

The emigration of inflammatory cells out of the vasculature in response to a wide variety of immunogens has been well described in chickens (Vegad and Katiyar, 1995). The leukocytic sequence appears stereotyped qualitatively regardless of the nature of inflammatory stimulus. The initial emigration of cells comprises heterophils and monocytes. This is soon followed by emigration of basophils, with lymphocytes appearing much later. After margination from the venules, heterophils and monocytes move from the perivascular areas to the location of immunogen and monocytes take on the appearance (increased cell size, irregular cell membrane, predominant Golgi and polyribosomes and increased hydrolytic enzyme content) and functional properties (phagocytosis) of macrophages. The concurrent emigration of heterophils and monocytoid cells and the participation of basophils appear to be the characteristic feature of the early inflammatory reaction in the chicken. In mammals, basophils do not appear to play a significant role in the development of acute inflammatory reaction. This suggests that basophils may be playing a specific role in the chicken as regulators of subsequent cellular changes, presumably through the release of histamine and possibly also 5-HT (Ito and Böhm, 1986; Klasing, 1991; Jones, 1994; Vegad and Katiyar, 1995). The mediation of the recruitment of heterophils in the peritoneum does not seem to be initiated by arachidonic acid metabolites. This was shown by Kogut et al. (1995) in an inflammation model where Salmonella enteriditis was administered in the peritoneal cavity. Another common feature of avian
inflammation seems to be the occurrence of perivascular lymphoid aggregation. This has been reported to occur in inflammation induced by a wide range of stimuli. Little is known, however, about their functional significance. They may play a role similar to mammalian lymph nodes. This is notable because lymph nodes are largely absent in chickens. A number of workers have described giant cell formation in the later stages of the acute inflammation in the chicken. These cells appear to be formed faster than in mammalian species, where they are formed only in the chronic phase of inflammation. In the chicken, eosinophils do not participate in the local inflammatory reaction induced by non-immunological stimuli (Vegad and Katiyar, 1995; Jones, 1994).

Reviews about morphology and functions of different leukocytic cell types in poultry have been published by several authors: macrophages (Golemboski 1990; Xie, 2001; Dietert, 1998; Klasing, 1998), heterophils (Harmon, 1998; Kogut et al., 1998; Maxwell, 1998), basophils (Maxwell, 1995) and eosinophils (Maxwell, 1987).

**ACUTE PHASE PROTEINS**

During the early phase of an inflammatory response in mammals, the concentrations of certain specific plasma (glyco)proteins, known as acute phase proteins (APP’s) change due to their increased or decreased synthesis by hepatocytes. Those whose synthesis is increased are called positive APP’s, whilst negative APP’s denote those proteins such as albumin whose synthesis is reduced. Acute phase proteins appear earlier than specific antibodies and decrease in a manner almost directly correlated with the resolution of the inflammatory response both in mammals and chickens. APP’s demonstrate the existence, but not the cause of inflammation. Inflammation can be caused by different stimuli, not only infectious. In domestic fowl, *E. coli* or its endotoxin has been used to induce the synthesis of α1-acid glycoprotein, ceruloplasmin, metallothionein and transferrin. Viral infections have been equally successful in inducing the synthesis of poultry APP’s. Infectious bronchitis, infectious laryngotracheitis and infectious bursal disease induced high levels of α1-acid glycoprotein. Also after parasitic infections, e.g. coccidiosis, a high level of ceruloplasmin has been reported. This could partly be due to a secondary bacteriosis and tissue injury caused by the parasite. Ceruloplasmin acts as an antioxidant to limit
the damage of free radicals released by macrophages (Klasing, 1991; Chamanza, 1999). Other non-infectious inflammatory conditions, e.g. injection of a chemical irritant, surgical trauma, burns and tumours, can induce an acute phase response. An injection of turpentine can increase the levels of transferrin, fibrinogen, fibronectin and haptoglobin. Transferrin is thought to deprive bacteria of free iron (Chamanza et al., 1999). Transferrin is a negative acute phase protein in mammals and its production and secretion in the liver decreases at acute phase. The behaviour of chicken transferrin is different from that of mammalian transferrin in the acute phase. The reason for this difference is unknown (Tohjo et al., 1995). Avidin seems to be induced in different chicken tissues and macrophages after inflammation caused by tissue injury and injection of \textit{E. coli}. This is a biotin binding protein first found in the egg white of the hen. Avidin inhibits the growth of biotin-requiring microbes (Elo, 1979; Klasing, 1991). Recently, ovotransferrin was found to be a major acute phase protein in chickens, although the significance in avian inflammation is not clear at this moment (Xie et al., 2002).

INFLAMMATORY MEDIATORS AND CYTOKINES

Inflammatory mediators and cytokines are implicated in the aetiology of a variety of processes underlying homeostasis and disease, including regulation of the immune response. Cytokines are peptide mediators not produced by a discrete endocrine gland, but rather by widely dispersed cells and tissues. Also, the actions of one cytokine are biologically very diverse (pleiotropic) and conversely, antigenically distinct cytokines may have similar or identical activities (redundant). They can include the induction of proliferation, cell death, anabolism, catabolism, activation, deactivation, chemotaxis, migration inhibition, differentiation or dedifferentiation. Cytokines regulate cellular activity in co-ordinated interactive cascades or networks where the release of one cytokine leads to the production and secretion of a series of others, resulting in either positive or negative feedback pathways (Kogut, 2000). In birds, much confusion about the existence and role of these cytokines is still going on. The identification is usually based on mammalian equivalents with similar bioactivities (Klasing, 1994). The exact inflammatory mediators elaborated in the initial stages of inflammation and their source have not been characterised in birds. In mammals they include the proinflammatory cytokines interleukin-1\textbeta (IL-1\textbeta), tumor necrosis factor-alpha (TNF-
α) and a wide range of other components: complement and their proteolytic fragments, interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), interferons, platelet-activating factor, heparin, histamine, leukotrienes and other arachidonic acid metabolites (Kogut, 2000; Van Miert, 2002). Together, these mediators act synergistically to induce local and systemic responses to the stimuli. The local responses include: (a) increased vascular permeability, (b) induced expression of adhesion molecules on vascular endothelium and (c) induced local production of chemokines. The systemic responses include fever, anorexia, gastric dysfunction, downregulation of albumin and drug metabolising enzymes, hepatic production of acute phase proteins and secretion of colony stimulating factors by endothelial cells, resulting in hematopoiesis and a transient increase in the required white blood cells to fight infections. In chickens, there is evidence of functional activity of IL-1, IL-6 and TNF that is homologous to the mammalian cytokines. Both type I (IFN-α/β) and type II (IFN-β) interferons have been cloned and to date, only one colony stimulating factor-like (CSF) substance has been identified and cloned: chicken myelomonocytic growth factor (cMGF). There is no mammalian homologue to cMGF (Klasing, 1991; Kogut, 2000). The species barrier has prevented the use of several cytokines of mammalian origin in avian systems and has often precluded the use of mammalian assay systems to identify avian cytokines. Many investigators have been unable to detect any activity of commercially available mammalian IL-1 or IL-6 in chicken assay systems (Klasing, 1991).

The use of exogenous cytokines against infectious agents in poultry medicine has focused on their use as adjuvants for vaccines, their direct effects on inducing protection against infections and their ability to stimulate the ontogeny and activation of neonatal host defences (Kogut, 2000).

CONCLUSION

Progress is made in different areas of inflammation research in birds. However, extensive work is required to further categorise the mechanisms of inflammation in birds and to look for possibilities to intervene pharmacologically in these mechanisms. Although there are similarities in the inflammatory reaction between
mammals and birds, there are also differences. Hence it is obvious that species specific research on inflammation is needed.
REFERENCES


A subcutaneous inflammation model in chickens

Adapted from:

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ABSTRACT

The current knowledge about inflammation in birds and the influence of non-steroidal anti-inflammatory drugs on inflammation is very limited. Sodium salicylate is an NSAID that possesses favourable pharmacokinetic properties in chickens and can be used to treat inflammations in birds. In this experiment, an acute inflammatory reaction was generated by implanting a carrageenan impregnated sponge strip in the subcutaneous tissue of broiler chickens. Half of the chickens received 50 mg/kg BW sodium salicylate orally and the other half received a placebo treatment. Blood and exudate samples were taken from these chickens at predetermined times. The pharmacokinetics of sodium salicylate were investigated in blood and exudate. Other parameters that were investigated were the volume of the exudate, the number of leukocytes in the exudate and the prostaglandin E\textsubscript{2} concentration in the exudate. Also bradykinin was injected intradermally and the effects of sodium salicylate on bradykinin induced oedema were studied. Maximum salicylic acid plasma concentrations occurred within the hour after administration and maximum exudate levels were seen at the first exudate sampling point (4 h). Salicylic acid exudate concentrations exceeded plasma concentrations at the same time points and the exudate half-life of elimination was longer than the plasma half-life of elimination. No differences were found in the volume of the exudate and the leukocyte numbers between the treated and the untreated group. Sodium salicylate reduced the PGE\textsubscript{2} concentration in the inflammatory exudate at the 4 hour time point, but at the later time points, no significant differences were found. The intradermally injected bradykinin did not produce significant effects in the chicken skin. Based on these findings, sodium salicylate may be used to suppress the inflammatory cascade in chickens, but further research is needed to characterise the inflammatory response in this chemical inflammation model and the actions of this non-steroidal anti-inflammatory drug in chickens.
INTRODUCTION

Classical non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed in different animal species and they often provide excellent symptomatic relief in many inflammatory disorders. The clinical signs associated with the process of inflammation have been recognised for thousands of years. Redness, swelling, heat, pain and loss of function can all be recognised to varying degrees in inflammatory foci in different species. The inflammatory response in tissues consists largely of an active vasodilatation with increased permeability of white blood cells (WBC). The protein-rich fluid and the cells which accumulate in the injured tissues following this process make up the inflammatory exudate which is a major feature of acute inflammation (May et al., 1987). Inflammatory exudate appears to have several functions which are relevant to subsequent healing. The fluid component dilutes any noxious substances present, thus reducing their harmful effect. The phagocytic actions of leukocytes lead to the destruction of infective micro-organisms and the removal of cell debris. Leukocytes are also a major source of the mediators involved in the chemotaxis of other WBC and the subsequent organisation of the inflammatory focus by fibroblasts and capillaries.

The process of inflammation may sometimes be inappropriate to a healing process, as in hypersensitivity reactions or too severe as to damage tissue. In these circumstances, a number of drugs are available to attenuate the symptoms and effects of inflammation. Sodium salicylate is one of the oldest NSAIDs and has been used for many years as an antipyretic, analgesic and anti-inflammatory agent in different species. This class of drugs inhibits symptoms associated with inflammation because they block the enzyme cyclo-oxygenase, which is necessary in the arachidonic acid cascade. This cascade forms an important group of inflammatory mediators, the eicosanoids such as prostaglandins and thromboxanes from arachidonic acid. In bird species there is a paucity on scientific research on pharmacokinetics and pharmacodynamics of NSAIDs. Also, the methods to investigate inflammation processes in chickens are limited. In this study, a model of non-immune inflammation, modified from Higgins et al. (1984) and Chansoriya et al. (1994), was used in chickens. Carrageenan, a mucopolysaccharide extract of Irish marine algae, was used to generate an inflammatory reaction. Since 1959, techniques using carrageenan were refined and these models became accepted as standard methods for screening and
CHAPTER 2.2

assessing the efficacy of anti-inflammatory drugs (Higgins et al., 1987). The objectives of this study were: the evaluation of the inflammatory effects of a mild subcutaneous inflammation model using sponges soaked in carrageenan in chickens, the time course of in vivo inhibition of synthesis of PGE$_2$ in inflammatory exudate and the effects of an anti-inflammatory drug on the formation of this eicosanoid inflammation mediator, and the pharmacokinetics of sodium salicylate in plasma and the penetration in the inflammatory exudate.

MATERIALS AND METHODS

Animals and experimental design
Twenty-four clinically healthy heavy breed chickens (1.8 ± 0.22 kg BW, Ross breed) were used for the study. The animals were kept in groups of four chickens and fed a commercial chicken feed (Duvo, Wondelgem, Belgium) along with tap water ad libitum. There was an acclimatisation period of 7 days before commencement of the study. Twelve chickens received a placebo treatment and 12 chickens received an oral administration of the salicylate solution at 50 mg/kg BW and this was already done 0.5 hour before the induction of the inflammatory stimulus. This was done by subcutaneous insertion of sponges. Only 2 subcutaneous pouches could be made on 1 chicken. To obtain a sufficient number of samples for each sampling point, we used 6 different chickens per point. The sponges were removed at 4, 8, 12 and 24 h.

Induction of inflammatory reaction
A mild inflammatory response was induced subcutaneously under the wing, above the pectoral muscle at both sides of each chicken. At this site, practically no feathers and feather follicles are present. No feathers were pulled to minimise traumatic dermal inflammation. An incision of 1.5 cm was made under local anaesthesia (2% lidocaine hydrochloride, Astra Pharmaceuticals, Brussels, Belgium). Care was taken not to cut visible blood vessels. Pressure was exerted until bleeding subsided (1 min) and a sponge strip (35 x 20 x 5 mm), treated with sterile 1% carrageenan (Sigma Chemical, St. Louis, MO, USA), was inserted in the subcutaneous pouch. The wound was not sutured for the duration of the study and was closed afterwards with 3 sutures. During
the study, asepsis was maintained by covering the wound with an adhesive non-woven fabric (Fixomull® stretch, Beiersdorf AG, Hamburg, Germany). Inhibition of bradykinin (BK)-induced swelling was evaluated as follows. Five µl of a 20 µg/ml solution of BK was injected intradermally in the sternal featherless region. After 30 minutes, the change in skin-fold thickness and 2 wheal diameters were measured, using spring gauge and vernier callipers, respectively. The volume of the wheal (assumed to approximate to half an ellipse) was calculated by applying the following equation: \[ V = \frac{2}{3} \pi r_1 r_2 r_3 \], where \( V \) is the volume of the wheal expressed in microlitres, \( \pi \) is the constant 3.1416, \( r_1 \) is the horizontal radius of the wheal, \( r_2 \) is the vertical radius of the wheal, and \( r_3 \) is the change in skin-fold thickness. Bradykinin was injected at 2 and 7 hours after administration of sodium salicylate.

*Collection of blood and exudate samples*

Blood samples for the collection of plasma were taken in the leg vein of six chickens at predetermined times up to 48 h into heparinised tubes. Plasma was collected after centrifugation (2500 x g, 10 min) and was stored at -20°C until analysis for salicylic acid concentration. The sponges containing acute inflammatory exudate were removed at 4, 8, 12 and 24 h after insertion. Exudate volume was measured after removal. From each sponge, inflammatory exudate was collected into tubes containing 70 IU heparine and 10 µg BW540C (Glaxo Wellcome Research and Development, Stevenage, Herts, UK), a dual cyclo-oxygenase 5-lipoxygenase inhibitor, to prevent artefactual in vitro generation of eicosanoids (Higgins and Lees, 1984). Following removal of a 0.1 ml aliquot, for measurement of leukocyte numbers, samples were centrifuged (2500 rpm, 10 min) to separate cells. The supernatants were divided into aliquots prior to storage at -20°C until analysis for PGE_2_ and salicylic acid concentration.

*Analytical methods*

Total leukocyte counts were done using a Coulter Counter (The Coulter Corporation, USA). PGE_2_ was analysed using a commercial ELISA method (Cayman Chemical, Ann Arbor, MI, USA). Salicylic acid and metabolites were quantitated using a HPLC method with a Focus Forward scanning UV-detector. Briefly, plasma concentrations of salicylic acid and two possible metabolites, gentisic acid and saliuric acid, were analysed on a Thermo Seperations Product (TSP, Fremont, CA, USA) HPLC-system.
using a P-4000 pump, Model AS 3000 autosampler and a Focus Forward scanning UV-detector set at 305 nm. A 250 x 4.6 mm I.D. reversed-phase column (5 µm Spherisorb ODS-2, Chrompack, Antwerp, Belgium) attached to an appropriate guard column was used. The injection volume was 100 µl. The mobile phase comprised 85% water-acetic acid (99:1, v/v) and 15% acetonitrile. A gradient solvent programme was run: 0-4 min : 85/15; 4-20 min : 85/15 – 60/40; 20.1-25 min : 85/15. The flow rate was 1 ml/min. Samples were prepared by pipetting 0.5 ml of plasma or exudate into a 15 ml screw-capped tube, followed by the addition of 50 µl of IS (o-anionic acid in methanol, 100 µg/ml), 150 µl of 1 M HCl and 5 ml of diethylether. After centrifugation (2400 rpm, 5 min), the organic layer was transferred to a clean screw-capped tube and evaporated under nitrogen at 40°C. The residue was redissolved in 250 µl of the mobile phase, briefly vortexed and 100 µl were injected.

Analysis of data
Plasma and exudate concentration time relationships were evaluated using compartmental pharmacokinetic methods. Plasma pharmacokinetic parameters were estimated by fitting the concentration data to an appropriate model by means of the MW/PHARM computer program (version 3.15, Groningen, The Netherlands). Values reported are mean ± SD. The significance of differences of means between drug and placebo treated animals was assessed by Student's t-test for unpaired data. The level of significance was 0.05.

RESULTS

Pharmacokinetics
The mean plasma and exudate concentrations of salicylic acid were plotted on a semi-logarithmic scale as a function of time and are shown in figure 1. The plasma curve was best described as a one compartment open model. The pharmacokinetic parameters obtained for salicylic acid are presented as the mean ± SD (n=6) in table 1. For the calculation of these parameters, a bioavailability of 80% was used, a value that was calculated in earlier experiments (Baert and De Backer, unpublished results).
Figure 1. Mean salicylic acid concentrations (± SD) in plasma (♦) and exudate (■) from broiler chickens after a single oral administration of a dose of 50 mg/kg BW (plasma n = 6, exudate n = 24).

The concentration of salicylic acid found in the exudate exceeds significantly the concentration found in plasma at the same time points and the half-life of elimination of salicylic acid in the exudate is approximately 7.29 h, which is longer than in the plasma.
Table 1. Pharmacokinetic parameters of salicylic acid (SA) in plasma after oral administration of 50 mg/kg of SA (n = 6, mean ± SD).

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<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>SA</th>
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<tr>
<td></td>
<td>MEAN</td>
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<tr>
<td>AUC (mg*h/l)</td>
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<tr>
<td>Cl (l/h*kg)</td>
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<tr>
<td>Vd(area) (l/kg)</td>
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<td>t1/2el (h)</td>
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<td>k_el (h^-1)</td>
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<td>C_max (mg/l)</td>
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<tr>
<td>T_max (h)</td>
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**Pharmacodynamics**

The volume of the exudate samples is presented in figure 2. No differences could be found between the samples of the untreated birds and the birds who received an oral bolus of 50 mg/kg of sodium salicylate.
Figure 2. Mean volume (ml) of exudate samples (± SD) from broiler chickens after a placebo (■) treatment or a single oral administration of SA (♦) at a dose of 50 mg/kg BW (n = 6 per sampling point).

The concentration of WBC in the exudate is presented in figure 3. No differences in the concentration of WBC in the treated or the non-treated group were found. The PGE₂ concentrations are presented in figure 4. The mean PGE₂ concentration was only significantly different between the salicylate treated birds and the placebo treated birds at the 4 hour point. Macroscopically, the injection of bradykinin intradermally gave a very mild reaction in the skin. A slightly paler colour of the skin at the site of injection was apparent in most of the chickens, but no changes in skinfold thickness could be shown.
DISCUSSION

Ideally, a model of inflammation should provide the investigator with the ability to monitor quantitatively clinical, physiological, cellular and biochemical aspects of the inflammatory response in the species of interest. In practice, however, it is difficult and perhaps impossible to achieve all of these wide-ranging objectives in a single model. Additionally, particular problems are encountered when studying the chicken. The chicken is a relatively small animal, and the skin is covered with feathers. Also the skin is very thin and the subcutaneous connective tissue is very loose and easily disrupted. Each of the 'cardinal' signs of inflammation, redness, heat, swelling, pain and loss of function can be used as an indicator of the progression of an inflammatory reaction.

Figure 3. Mean concentration of WBC in the exudate samples (± SD) from broiler chickens after a placebo (■) treatment or a single oral administration of SA (♦) at a dose of 50 mg/kg BW (n = 6 per sampling point).
Figure 4. Mean concentration of PGE$_2$ in the exudate samples (± SD) from broiler chickens after a placebo (■) treatment or a single oral administration of SA (♦) at a dose of 50 mg/kg BW (n = 6 per sampling point).

In chickens, few inflammation models have been used and information on efficacy of anti-inflammatory drugs is very scarce. Studies on the increase of vascular permeability after chemically induced inflammation (turpentine, carrageenan) were done by Ito and Böhm (1986) and Jain et al. (1995). One research group recently developed an intra-articular inflammation model with sodium urate crystals in chickens. In this model, behavioural observations were done and were linked to the analgesic properties of the drug tested (Gentle, 1997; Hocking et al., 1997). Others used naturally occurring musculo-skeletal disorders to evaluate anti-inflammatory drugs (Mc Geown et al., 1999). In this model, the classical signs of inflammation could not easily be assessed. Redness and heat could not be measured because of the anatomical features of the chicken skin: very thin and feathered, furthermore, the site of inflammation was situated under the wing. Swelling of the lesion could not be measured because the size of the inflammation site was largely dependent on the size of the foreign body. An attempt to measure the increased vascular permeability was done by assessing the volume of exudate and the number of leukocytes as a measure for diapedesis. Pain and loss of function are difficult to assess in this model in birds.
Current methods to assess pain are based on behavioural studies. In this study, no detailed behavioural observations were done.

There is also the question of the ethical applicability of any model developed for use in domestic animal species. Practically all models that investigate inflammation cause pain and distress to the animals. A principal objective in designing new models, therefore, was to minimise any distress and discomfort to the animals being used. In this study, chickens seemed to tolerate the procedure well. No stress reactions were noted during the incision and insertion of the foreign body and during the study, chickens showed a normal feeding and drinking behaviour. After the study, wounds were closed and healing was rapid and complete.

Pharmacokinetic parameters after oral administration of sodium salicylate are already described for different animal species, including dogs (Waters et al., 1993), cattle and goats (Short et al., 1990a) and rabbits (Short et al., 1990b). In chickens, very few reports are available about plasma concentrations after oral administration of sodium salicylate (Nouws et al., 1994). The findings in this chapter show a half-life of 6.07 h, which is slightly longer than the half-life found after intravenous administration (4.04 h) of 50 mg/kg sodium salicylate in an earlier experiment. This could be due to delayed absorption processes or a saturation in the formation process of a metabolite. However, a generally fast absorption was found with a $T_{\text{max}}$ after 0.30 h and a $C_{\text{max}}$ of about 40 mg/l. The exudate concentrations exceed the concentrations in the plasma significantly at the same time points. Also a longer half-life of elimination was seen in the exudate (figure 1). The present study confirms that salicylic acid penetrates into and is slowly cleared from inflammatory exudate in the chicken. This finding is in accordance with published results in other species and other NSAIDs (Landoni and Lees, 1995; Scherkl et al., 1996; Lees et al., 1999).

The exudate samples were practically uncontaminated with blood. This is in contrast with the data obtained in horses by Higgins and Lees (1984). The volume of the exudate samples are shown in figure 2. Volumes rose from 3 ml at the 4 hour point to more than 10 ml at 24 hours. However, no difference could be shown between the sodium salicylate treated birds and the placebo treated birds. As indicated by figure 3, leukocyte infiltration into exudate occurred following the insertion of the carrageenan treated sponges. Cell numbers rose at 4, 8, 12 and 24 hours. Cells were relatively slow to mobilise but by 12 hours, numbers were still rising and at 24 hours the mean count had almost reached $10 \times 10^9$ cells/litre. Higgins et al. (1987) found a value of 100 x
10^9 cells/litre in the equine sponge model. The somewhat lower number of cells could be due to the less vascularised subcutaneous tissue and the fewer number of macrophages present in the chicken. Again, no difference could be shown between the sodium salicylate treated birds and the placebo treated birds. Thus, a single oral administration of 50 mg/kg sodium salicylate previous to the inflammatory stimulus seems to have no influence on chemotaxis into the inflammatory exudate. This was also the case for 0.5 mg/kg of meloxicam in an identical study design in chickens (Baert and De Backer, unpublished results). In experiments with other animal species, e.g. in rat and equine carrageenan induced inflammation models, phenylbutazone or flunixin could not inhibit leukocyte accumulation (Higgs et al., 1980 ; Lees and Higgins, 1984). Earlier experiments in rats, however, showed a depression of leukocyte migration into plastic sponge exudate in rats after administration of 150 mg/kg aspirin (Di Rosa, 1979). This could mean that a dose of 50 mg/kg might be insufficient to obtain an effect on the migration of leukocytes.

The concentration of the eicosanoid PGE_2 in the exudate was relatively low in comparison with findings by Higgins et al. (1987). They found values ranging from 5-60 ng/ml. This could be due to the nature of the tissue that was stimulated. In the chicken, the subcutaneous tissue is much less vascularised and fewer macrophages are present. A major difference between the chicken and the horse data is seen in the shape of the curve. In chickens, only in the first time point (4 h) a significant difference could be found between the sodium salicylate treated birds and the placebo treated birds. In horses the maximal concentration of PGE_2 is found at 12 hours. This could mean that in chickens, the role of the eicosanoids in this inflammation model is restricted to the first hours after the inflammatory stimulus. This is in accordance with the findings of Ito and Böhm (1989). They concluded that prostaglandin mediation of inflammatory oedema after carrageenan induced foot pad oedema occurred to some degree in the later stages of the inflammation (2.5-5 hours after the inflammatory stimulus). Since maximal numbers of leukocytes and maximal concentration of eicosanoids do not coincide, these data suggest that other cells or tissues may be involved to a greater extent in the synthesis of these compounds.

Bradykinin injected intradermally induced oedematous lesions, with peak volumes at 30 minutes, in calves and horses. The mechanism of action is unknown, but it may be cyclo-oxygenase dependent, because BK is known to release inflammatory eicosanoids through activation of phospholipase A2 (Landoni et al., 1995 ; Auer et al.,
1991; Dray and Perkins, 1993). Thus, treatment with an NSAID can attenuate the swelling caused by BK, as has been shown by Landoni et al. (1995) for flunixin in calves. In this experiment in chickens, practically no swelling was seen, only a paler colour of the skin at the site of injection of most of the chickens. Bradykinin induced changes in vascular permeability in the chicken skin were studied by Awadhiya et al. (1980). These researchers found that the venular response to BK was slightly delayed up to two hours after injection and not as strong as the response to histamine.

CONCLUSION

This inflammation model in chickens has advantages and disadvantages. Only a few characteristics of inflammation can be assessed and none of the cardinal signs of inflammation can easily be measured in this model. Based on the present findings, sodium salicylate may be used to suppress the inflammatory cascade in chickens, but further research is needed to characterise the inflammatory response in this chemical inflammation model and the actions of the non-steroidal anti-inflammatory drug in chickens.

ACKNOWLEDGEMENTS

The authors thank Prof. Dr. P. Lees for providing the BW540C, Prof. Dr. E. Meyer for the use of the coulter counter and Mrs. H. Lippens for her quality technical assistance.
REFERENCES


CHAPTER 2.3

An intravenous LPS inflammation model in chickens

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ABSTRACT

The use of non-steroidal anti-inflammatory drugs in bird medicine is receiving increasing interest, but its pharmacodynamic characteristics in birds are largely unknown. Therefore, two experiments were set up with sodium salicylate, a typical and often used non-steroidal anti-inflammatory drug, to study the effects of this NSAID upon the acute phase reaction induced by an intravenous injection of *Escherichia coli* lipopolysaccharide. In the first experiment, different oral doses of sodium salicylate were given and the effect on body temperature was measured. Other inflammation indices, such as plasma corticosterone and ceruloplasmin levels, serum thromboxane B₂ and zinc levels were also monitored during the experiment. In a separate experiment, food and water consumption and other behavioural parameters were studied. In the first experiment, a dose dependent attenuation of the fever response of the chickens in the salicylate treated groups was observed. Plasma corticosterone and ceruloplasmin levels rose and zinc and thromboxane B₂ levels declined after an LPS injection. Except for thromboxane B₂, no dose dependent differences after treatment with sodium salicylate were seen in these parameters. In the second experiment, the water intake, but not the food intake, was significantly higher in the salicylate treated group. No behavioural differences were seen between the positive control and the salicylate treated group. These data confirm that sodium salicylate is an effective antipyretic agent after injection of LPS in chickens, if used at an appropriate dosage.
CHAPTER 2.3

INTRODUCTION

Salicylic acid is found in some plants and has been used for more than 2000 years as an anti-inflammatory, antipyretic and analgesic agent in man and many animal species. Sodium salicylate, a water soluble salt of salicylic acid, is frequently used in the poultry industry. Several indications for the use of non-steroidal anti-inflammatory drugs (NSAIDs) have been proposed, including respiratory diseases, digestive coccidial and bacterial infections, inadequate intestinal equilibrium to sustain good weight gain, broiler ascites, heat stress, locomotion disturbances and stimulation of egg production and eggshell quality (Thomas et al., 1966; Proudfoot and Hulan, 1983; Jouglar and Bernard, 1992; Balog and Hester, 1991; McDaniel et al., 1993; Shlosberg et al., 1996; Abd-Ellah et al., 1997; Mc Geown et al., 1999; Cristòfol et al., 2000). For many of these indications, the benefits of an NSAID treatment have not been clearly proven or relevant experiments led to negative results. It is well known that NSAIDs act as inhibitors of prostaglandin synthesis and are therefore used as antipyretics, analgesics and anti-inflammatory agents (Vane and Botting, 1995). Despite an increasing awareness of animal suffering and the recognition of medical, traumatic and surgical conditions causing pain in animals, there are relatively few pharmacodynamic data on non-steroidal anti-inflammatory drugs in poultry. Nevertheless, analgesics are routinely required for clinical use and there are some recommendations on appropriate analgesics for avian species, apparently based on practical experience (Hocking et al., 1997). Therefore it is necessary to perform pharmacodynamic studies in the target animal. Together with the pharmacokinetic information, a more precise calculation of the required dosage and dosing interval can be made.

The administration of an intravenous dose of endotoxin (lipopolysaccharide, LPS) of Salmonella, Shigella, and especially Escherichia coli has been investigated in chickens by many authors (Smith et al., 1978; Artunkal et al., 1977; Butler et al., 1978; Jones et al., 1981; Macari et al., 1993; Johnson et al., 1993; Nakamura et al., 1998; Leshchinsky and Klasing, 2001). LPS is less lethal in fowl compared to mammals, but a period of pyresis and several behavioural, metabolic and endocrine changes, known as the acute phase reaction, occur after the injections of small quantities of LPS in chickens (Smith et al., 1978; Butler et al., 1978; Jones et al., 1981). The mechanism for this apparent difference in sensitivity to LPS between
mammals and aves has yet to be elucidated, however, a possible explanation could be that uric acid has been demonstrated to bind LPS and protect against endotoxin shock. Because avian species have relatively high circulating plasma levels of uric acid, it is possible that this difference may account for a reduced sensitivity to LPS (Adler and DaMassa, 1979; Koutsos and Klasing, 2001). This study provides information on the effect of different doses of orally administered sodium salicylate on an intravenous \textit{E. coli} lipopolysaccharide acute phase reaction in broiler chickens. The different parameters (body temperature, corticosterone, ceruloplasmin, zinc, behaviour, feed and water consumption) were selected from the literature on LPS acute phase reactions in chickens. Based on the general fever mechanism and the mechanism of action of NSAIDs, one can expect an effect of sodium salicylate on the body temperature of these LPS injected chickens. Except for thromboxane B\textsubscript{2}, other parameters (corticosterone, ceruloplasmin and zinc) are not directly linked with a prostaglandin dependent mechanism, but were also measured during the experiment to assess the severity of the acute phase reaction. Like in mammals, the decrease in feed consumption and behavioural changes after an LPS injection may be reversed with an NSAID treatment.

MATERIALS AND METHODS

\textit{Animals and experimental protocol}  
Two experiments were carried out with groups of clinically healthy male broiler chickens (Ross) weighing 1.4 ± 0.3 kg. The daily photoperiod was 12 h and ambient temperature was maintained at 22-23°C.

\textit{Experiment 1}: The animals in the first experiment were assigned to six groups of 10 chickens and were fed a commercial chicken feed (Duvo, Wondelgem, Belgium) and tap water was provided \textit{ad libitum}. One group served as an untreated control group. One group received \textit{E. coli} LPS intravenously, and four groups received \textit{E. coli} LPS intravenously and were treated 30 min before LPS administration with an oral bolus administration of sodium salicylate at a dose of 25, 50, 100 or 200 mg/kg BW, respectively. LPS was administered by intravenous bolus injection in the wing vein at a dose of 1 mg/kg. Rectal temperatures to the nearest 0.1°C were taken at 0, 1, 2, 3.5, 5, 6.5, 8, 12 and 24 h with a thermocouple probe (RET-1) attached to a temperature recorder (Model BAT-12) from Physitemp Instruments Inc. (Clifton, USA). The
probe was inserted 5 cm deep in the rectum of the chicken and the reading was taken after one minute of contact with the mucosa. Blood samples from six birds per group were collected in heparinised tubes (Venoject®, Terumo Corp., Tokyo, Japan) from the vena metatarsalis communis at 2 and 24 h for determination of corticosterone and ceruloplasmin levels, respectively, and in serum tubes at 2 and 12 h for determination of thromboxane B₂ (TxB₂) and zinc levels, respectively. The serum tubes for thromboxane analysis were allowed to cloth for 1 hour at 40°C. Plasma and serum were separated by centrifugation (2400 rpm for 15 min at 5 °C) and the samples were stored at -20 °C until assayed.

**Experiment 2**: The animals of the second experiment were housed in 6 groups of 5 chickens. Two groups received *E. coli* LPS intravenously, two groups received *E. coli* LPS intravenously and were treated with an oral bolus administration of sodium salicylate at a dose of 100 mg/kg 30 min before LPS administration and two groups served as untreated controls. Consumption of food and drinking water were weighed per group and recorded at 0.5, 1, 1.5, 2, 3, 4, 5, 8 and 12 h for each group. The birds were fasted for a few hours before the administration of the LPS and food was given 1 hour after administration of the LPS. The collection of the other behavioural parameters was initiated after feeding the birds.

**Drugs and reagents**

Commercially available *E. coli* LPS (O127:B8, Sigma, Bornem, Belgium) was dissolved in NaCl 0.9% and used for intravenous injection. Sodium salicylate, obtained from Sigma, was dissolved in tap water and used for oral administration. Standards for corticosterone and prednisolone (internal standard (IS) for the corticosterone method) were obtained from Sigma and the manufacturing company, respectively. Solvents of high-performance liquid chromatography (HPLC) grade were obtained from Sigma and Acros (Geel, Belgium) and were used for HPLC analysis. o-Dianisidine (ODA), morpholino-ethane sulfonic acid (MES) and H₂SO₄ for ceruloplasmin analysis were also obtained from Sigma.

**Determination of corticosterone and ceruloplasmin in chicken plasma**

Corticosterone levels were determined in chicken plasma samples as follows. A 500 µl plasma sample was transferred into a glass extraction tube of 5 ml and 50 µl of a 2 µg/ml working solution of prednisolone (IS) were added. After vortex mixing for
15 s, 3 ml of diethylether were added. The tube was rolled for 20 min and then centrifuged (2500 rpm, 5 min, 5°C). The upper etherphase was transferred into a new extraction tube and dried under a gentle stream of nitrogen at 40 °C. The dry residue was then dissolved in 150 µl of the mobile phase A and a 50 µl aliquot was injected onto the LC column. The LC column used was a reversed phase C<sub>18</sub> Nucleosil column (5 µm, 100 mm x 3 mm ID), in combination with a guard column of the same type (2.5 cm x 3 mm ID), both from Chrompack (Middelburg, The Netherlands). The mobile phase A was a solution of 1 % acetic acid in water, while the mobile phase B was acetonitrile. The flow was set at 0.2 ml/min for a total run time of 20 min. A gradient elution was used: 0-1 min: 70 % A, 30 % B; 1.1-9 min: 30 % A, 70 % B; 9.1-20 min: 70 % A, 30 % B. Corticosterone eluted then at 8.4 min, and prednisolone at 7.1 min. The LC effluent was pomp to an LCQ<sup>®</sup> ion trap mass spectrometer instrument (Finnigin MAT, ThermoQuest, San Jose, CA, USA), equipped with an ESI ion source, used in the positive ion MS/MS mode. The instrument was calibrated according to manufacturers instructions and tuned with a 10 µg/ml solution of corticosterone. Tune parameters retained for optimal corticosterone detection were also found to be suitable for prednisolone detection. Corticosterone was found in MS mode as its protonated molecular ion [M+H]<sup>+</sup> at m/z= 347.2 and presented in the MS/MS mode product ions at m/z= 329.1, 311.2 and 293.1 by successive losses of H<sub>2</sub>O, at a collision energy of 1.3 V. In the same way, prednisolone was found in MS at m/z= 361.1 and produced in MS/MS product ions at m/z= 343.1, 325.1 and 307.1. Quantification was done with the LCQuan<sup>®</sup> software (ThermoQuest), using the m/z= 293.1 product ion for corticosterone, and the m/z=325.1 product ion for prednisolone. Linear calibration curves were obtained in the 0-100 ng/ml range. Precision and accuracy (n=6) were satisfactory at 20 ng/ml and 2 ng/ml (= limit of quantification (LOQ)).

Ceruloplasmin was measured spectrophotometrically according to Kim and Combs (1988). Briefly, for each sample, two tubes were prepared by the addition of 0.75 ml 0.1 M MES, pH 6.1, and 50 µl of the plasma sample to each tube. The tubes were pre-incubated at 40°C for 5 min, after which additions of 0.2 ml ODA reagent (2.5 mg o-dianisidine dihydrochloride/ml deionized water, w/v) were made to each. The complete incubation mixtures were held at 40°C for either 10 min (blank) or 60 min (sample), at which times the reactions were terminated by adding 2 ml 9 M H<sub>2</sub>SO<sub>4</sub> to each. The colour, which is stable for at least 90 min, was quantitated by measuring its
absorbance at 540 nm on a spectrophotometer (model Genesys 10uv, Spectronic Unicam, Rochester, USA). Ceruloplasmin activity was calculated as follows: ODA oxidized µmoles/min/litre = \((A_{600} - A_{10}) \times 60 \times 1000 \text{ ml}) / (9.6 \text{ ml} \times \mu\text{mole}^{-1} \times \text{cm}^{-1} \times 50\text{min} \times 1 \text{ cm} \times 1 \text{ litre})

**Determination of thromboxane B\(_2\) and zinc in chicken serum**

The analysis of thromboxane B\(_2\) was done with a commercial Enzyme Immunoassay Kit obtained from Cayman Chemical company (Ann Arbor, USA). Analysis was done according to the instructions provided by the manufacturers. They report a 100% specificity, cross-reaction of 9.9% with 2,3-dinor-thromboxane B\(_2\), and <1% cross-reaction with prostaglandins D\(_2\), E\(_2\), F\(_1\alpha\), F\(_2\alpha\), 6-keto prostaglandin F\(_1\alpha\), 2,3-dinor-6-keto prostaglandin F\(_1\alpha\), 13,14-dihydro-15-keto prostaglandin F\(_2\alpha\), 11-dehydro-thromboxane B\(_2\), and leucotriene B\(_2\).

Zinc was quantified *in duplo* in the chicken serum using an atomic absorption spectrophotometer (Perkin-Elmer Model 460, Norwalk, CT) using a 5-s integration period. The samples were diluted 1:4 with distilled, deionized water. Sample concentrations were calculated based upon comparison to a standard curve produced by dilution of a 1000-ppm reference solution (Fisher, Pittsburgh, PA; #SZ13-500), using distilled, deionized water.

**Behavioural observations**

The food and water consumption was measured. The birds were observed for three hours by an observer unaware of the treatment assigned to the birds. Bird activity was recorded every ten minutes as standing, walking, feeding, drinking, grooming, resting awake, resting with eyes closed, dustbathing and grooming while resting.

**Analysis of data**

The statistical analysis of the effect of LPS and NSAIDs on temperature was based on the mixed model (SAS, version 8) with chicken included as random effect in order to account for the repeated measures structure of the data. Time was divided into 3 periods: period 1 corresponds to the time just before LPS administration, period 2 to the time from 1 to 5 hours after LPS administration and finally period 3 to the time from 6 to 8 hours after LPS administration. First, a mixed model was fitted to
compare no treatment with LPS administration only in periods 2 and 3 to ensure that LPS had a significant effect on temperature (positive control). Next, a mixed model was fitted to the LPS only and LPS with different NSAID concentrations treatment groups with the NSAID concentration included as a continuous variable with a unit change for each doubling of the concentration (0 mg/kg=0, 25 mg/kg=1, 50 mg/kg=2, 100 mg/kg=3, 200 mg/kg=4). The linear relationship between the NSAID concentration and the temperature was assessed and tested for in periods 2 and 3. Corticosterone, ceruloplasmin, thromboxane B₂ and zinc were only measured once for each chicken and the analysis was thus based on the fixed effects model. First, no treatment was compared to LPS as positive control, followed by an assessment of the linear relationship between the NSAID concentration and each of these 4 variables. The analysis of feed and water intake was based on a fixed effects model on the cumulative feed and water intake per group up to 5 hours after treatment administration. Pairwise comparisons between the 3 treatment groups were performed based on Tukey’s multiple comparisons method with family confidence coefficient equal to 95%.

The behaviour was categorized into an ordered variable as follows: 0 = resting with eyes closed; 1 = resting awake, grooming while resting; 2 = eating, standing, walking, feeding, drinking, grooming, dustbathing. This ordered variable was averaged over time and group, and the statistical analysis was based on the fixed effects model on these average values with pairwise comparisons between the 3 treatment groups based again on Tukey’s multiple comparisons method with family confidence coefficient equal to 95%.

RESULTS

The average body temperature of the chickens for the negative control group, the positive control group and the 4 treatment groups (25, 50, 100 and 200 mg/kg) are shown in figure 1. An IV injection of LPS produces an increase in body temperature as compared to the negative control group, both between 1 and 5 hours (+0.68 °C, P<0.0001) and between 6 and 8 hours (+0.71 °C, P<0.0001). The NSAID concentration has a significant effect on temperature in the period from 1 to 5 hours after LPS administration: with each doubling of the NSAID concentration, temperature decreases 0.33 °C (P<0.0001), whereas in the period from 6 to 8 hours...
after LPS administration, this relationship is much less pronounced with a decrease of 0.07 °C (P=0.056) with the doubling of the NSAID concentration. The body temperature during the first period for the two highest SA doses was even lower than the negative control group.

![Temperature Change Graph](image)

Figure 1. Average body temperature of chickens (n=10 per group) after intravenous administration of lipopolysaccharide (lps), a control group (blank), and after IV administration of LPS and oral treatment with sodium salicylate at a dose of 25, 50, 100 and 200 mg/kg (sa25, sa50, sa100, sa200).

The corticosterone results are shown in figure 2. Although the LPS only group has a higher corticosterone level at 2 hours after LPS administration than the negative control group (P<0.0001), no linear relationship between the NSAID concentration and the corticosterone concentration could be demonstrated. No corticosterone concentrations above the LOQ (2 ng/ml) could be detected in the negative control group.
Figure 2. Average plasma corticosterone concentrations of chickens (n=6 per group, ± SD) at 2 hours after intravenous administration of lipopolysaccharide (lps), a control group (blank), and after IV administration of LPS and oral treatment with sodium salicylate at a dose of 25, 50, 100 and 200 mg/kg (sa25, sa50, sa100, sa200).

The ceruloplasmin activity measured at 24 h after injection of the LPS is plotted in figure 3. No significant linear relationship is seen with increasing NSAID dosis, although the LPS only group has far higher ceruloplasmin activity than the negative control group (P<0.0001).
Figure 3. Average plasma ceruloplasmin levels ($\mu$mol/min/l) of chickens (n=6 per group, ± SD) at 24 hours after intravenous administration of lipopolysaccharide (lps), a control group (blank), and after IV administration of LPS and oral treatment with sodium salicylate at a dose of 25, 50, 100 and 200 mg/kg (sa25, sa50, sa100, sa200).

The serum thromboxane concentration is shown in figure 4. A significant linear relationship of the TxB$_2$ decline with the dose of the NSAID is found. With each doubling of the NSAID concentration, the TxB$_2$ concentration decreases 2.11 pg/ml ($P=0.0028$). However, it was curious to find such a low level of TxB$_2$ in the positive control group which differed significantly from the negative control group ($P<0.0001$).
Figure 4. Average serum thromboxane B2 levels of chickens (n=6 per group, ± SD) at 2 hours after intravenous administration of lipopolysaccharide (lps), a control group (blank), and after IV administration of LPS and oral treatment with sodium salicylate at a dose of 25, 50, 100 and 200 mg/kg (sa25, sa50, sa100, sa200).

The serum zinc levels are shown in figure 5. No significant linear relationship is seen with increasing NSAID dosis, but the LPS only group has a lower zinc level than the negative control group (P=0.012).
Figure 5. Average serum zinc levels of chickens (n=6 per group, ± SD) at 12 hours after intravenous administration of lipopolysaccharide (lps), a control group (blank), and after IV administration of LPS and oral treatment with sodium salicylate at a dose of 25, 50, 100 and 200 mg/kg (sa25, sa50, sa100, sa200).

The intravenous injection of LPS has profound effects on the sickness behaviour. For the food consumption, a statistical significant difference is seen between the negative control group and the LPS-treated groups, but not between the positive control group and the NSAID-treated group. This is shown in figure 6.
Figure 6. Consumption of feed of chickens (n=5 per group) after intravenous administration of lipopolysaccharide (lps), a control group (blank), and after IV administration of LPS and oral treatment with sodium salicylate at a dose of 100 mg/kg (sa100).

For the drinking water consumption however, a significant difference is found for all pairwise comparisons. The amount of water consumed by the NSAID-treated group (485.5, 95% CI: 467-505) is intermediate to the negative (713, 95% CI: 694-733) and positive control group (218, 95% CI: 199-237). This is shown in figure 7.
Figure 7. Consumption of water of chickens (n=5 per group) after intravenous administration of lipopolysaccaride (lps), a control group (blank), and after IV administration of LPS and oral treatment with sodium salicylate at a dose of 100 mg/kg (sa100).

For the behaviour, again a statistical significant difference is seen between the negative control group (1.55, 95% CI: 1.25-1.85) and the NSAID-treated group (0.53, 95% CI: 0.23-0.83) (P<0.0001) but not between the positive control group and the NSAID-treated group (0.72, 95% CI: 0.42-1.02) (P=0.65). This is shown in figure 8. The groups that received LPS became visibly ill within an hour after LPS injection: they became recumbent and somnolent (eyes closed) and reluctant to walk and eat. Liquid excreta appeared after about 1 hour in some birds.
Figure 8. Mean behavioural observations (± SD) of chickens (2 groups (n=5 per group) per behavioural category (0, 1 and 2)) after intravenous administration of lipopolysaccharide (lps), a control group (blanc), and after IV administration of LPS and oral treatment with sodium salicylate at a dose of 100 mg/kg (sa100).

DISCUSSION

Body temperature
The chickens were injected intravenously with *E. coli* LPS. The serotype O127:B8 and the dosage were chosen on the basis of earlier experiments by other authors (Jones *et al.*, 1981; Brown-Borg and Edens, 1991; Freifeld *et al.*, 1995). Although recent findings suggest a lower febrile response to intra-abdominally injected *S. typhimurium* in broiler chickens compared to layer chickens (Leshchinsky and Klasing, 2001), our experiments showed a mean body temperature rise of about 1°C after the injection of 1 mg/kg *E. coli* LPS. However, not all the birds showed fever of the same magnitude, some animals seemed to have a less intense response and their body temperature only rose 0.4-0.5 °C. Some authors observed a short period (< 60 min) of hypothermia preceding the hyperthermia in young chickens (1-3 weeks of age). The same was observed in mammalian species and Japanese quail (Jones *et al.*, 1983; Leshchinsky and Klasing, 2001). In our experiment, similar to other studies
(Jones et al., 1983; Macari et al., 1993), the hypothermia was not observed in the positive control group. This variation in initial body temperature may be due to different factors, e.g. age of the birds (6-8 weeks of age), the route of administration of the LPS, the type of LPS, the study design, the restraint of the chickens and the environmental conditions (Dogan et al., 2000). The dip in the temperature curve at 5 hours could mean that the fever response is biphasic. This is a typical feature of several mammalian models. This was also shown by Jones et al. (1981) in young broiler chickens. However, other authors did not report a biphasic response in chickens (Johnson et al., 1993b; Macari et al., 1993) or Pekin ducks (Maloney and Gray, 1998) or Japanese quails (Koutsos and Klasing, 2001). The dip in the temperature curve at 5 hours could also be due to the feeding of the chickens at this time. Chickens are stimulated to eat and drink and this could cause a lower body temperature. Teeter et al. (1987) found that water consumption is negatively correlated with body temperature rise during broiler heat exposure. During heat stress, water plays a fundamental role in the evaporative heat loss mechanisms involved in the thermoregulation of the birds (Furlan et al., 1998).

In this LPS experiment, a dose dependency in attenuating the fever response is seen. With each doubling of the NSAID concentration, temperature decreases 0.33 °C (P<0.0001), and the highest dose effectively attenuates the fever for the first 5 hours. It seems that the effects of the NSAID only last until 5 hours after LPS administration. This could be caused by the pharmacokinetic properties of the drug. The plasma concentration of salicylic acid for which effective antipyretic, anti-inflammatory and analgesic activity can be achieved, has been reported to be 50 µg/ml for several species (Lees and Higgins, 1985; Lees et al., 1991). After a single intravenous dose of 25 mg/kg, the plasma salicylate levels did not reach 50 µg/ml (chapter 1.2.). After a single intravenous dose of 50 mg/kg, the plasma salicylate levels exceed 50 µg/ml for approximately 5 h and the mean half-life of sodium salicylate after an IV dose of 50 mg/kg was 4.04 h (Baert and De Backer, 2002), which means that salicylic acid levels are maintained in the plasma for a reasonably long period of time. After an oral dose of 50 mg/kg the maximal plasma salicylic acid concentration was about 40 µg/ml and the half-life was 6.07 h (chapter 2.2). Another possible reason could be that the first and the second phase of the biphasic fever in chickens are mediated by different systems. It is possible that the first phase is mainly mediated by eicosanoid products which are inhibited by NSAIDs. This was already
hypothesised by Skarnes et al. (1981) in their work with LPS infusions in sheep. They state that the prostaglandin E (PGE) is the main mediator of the early phase and that an additional pyrogenic derivative of the arachidonic acid metabolism is responsible for the second phase of the biphasic response. Moreover, Morimoto et al. (1987) found in their intravenous LPS model in rabbits that after subcutaneous treatment with indomethacin only the first phase of the fever response was suppressed. Recent attempts to clarify the mechanism of the second temperature rise in biphasic fever were not successful and Romanovsky and Blatteis (1995) concluded that various pyrogenic cytokines are responsible. The body temperature of the groups treated with the two highest doses during the fever period was even lower than the control group. This was already shown by D'Alecy and Kluger (1975) in pigeons. When they injected dead Pasteurella multocida, fever developed, which was effectively abolished by sodium salicylate. In these NSAID-treated pigeons, temperature also fell to a value lower than the normal values of control pigeons. In another experiment, they showed that pigeons which only received a treatment with sodium salicylate developed these sub-normal body temperatures. This was also shown in chickens by Pittman et al. (1976) and Cabanac and Aizawa (2000). They found that after administration of salicylate, the body temperature was at least 0.4°C lower than the control group. The administration of sodium salicylate in non-febrile rats exposed to cool or neutral ambient temperatures also resulted in a decline in body temperature (Satinoff, 1972 ; Solomonovich and Kaplanski, 1985). The mechanism of this temperature decline is not fully understood, but it may be related to a mechanism that is not associated with prostaglandin cyclo-oxygenase inhibition, possibly a direct action on the central nervous system (Solomonovich and Kaplanski, 1985 ; McCain and Mundy, 1987). Furthermore, Green and Lomax (1973) stated that it is unlikely that this hypothermic action of sodium salicylate in rats plays any part in the reversal of fever. But in other species (rabbits and humans), no temperature decline after the administration of salicylates in non-febrile subjects was found (Pittman et al., 1976 ; Rosendorff and Cranston, 1968). Also, a preliminary experiment at our laboratory in non-febrile broiler chickens could not reproduce the temperature decline after the oral administration of 200 mg/kg sodium salicylate (Baert and De Backer, unpublished results). There is still no consensus on the mechanism of fever in birds and the mediators which are responsible for this general inflammation symptom. Nevertheless, it is
suggested by several authors that the basic mechanisms of fever are similar in birds and mammals (Johnson et al., 1993; Leshchinsky and Klasing, 2001). Prostaglandins are found in the avian brain and hyperthermia in pigeons and chickens can be produced after an experimental injection of PGE into the hypothalamus (Nisticò and Marley, 1973; Pittman et al., 1976, Macari et al., 1993). However, other authors demonstrated that below thermoneutrality, a hypothermia was produced after infusing PGE in the hypothalamic area (Artunkal et al., 1977). Also, administration of E. coli LPS produced a rise in body temperature but no increase in the ex vivo hypothalamic PGE₂ production in domestic fowl (Fraifeld et al., 1995). These contradictory findings support the hypothesis that the hypothalamus should primarily be considered as an integrator of inputs from the spinal cord, brain and periphery, rather than a sole controller of thermoregulatory response in birds (Barnas and Rautenberg, 1987). The role of prostaglandins in avian fever obviously needs further study. If avian fever is ultimately PG-mediated then perhaps it is via PG from extrahypothalamic sources (Maloney and Gray, 1998). Furthermore, the mechanism of fever in mammals is influenced by multiple endogenous mediators, such as interleukin-1β, interleukin-6 and tumor necrosis factor-α. Therefore, efforts should be made to characterise the avian analogues of these mediators. These mediators activate the hypothalamic-pituitary-adrenal axis and mediate systemic components of the inflammatory response such as fever, sickness behaviour, anorexia, and acute phase protein release (Leshchinsky and Klasing, 2001).

Corticosterone

Corticosterone is the most important adrenocortical hormone in chickens and its plasma concentration is widely used as an indicator of stress. Following an LPS induced acute phase reaction, a maximal rise in corticosterone levels at 2 h after injection can be expected (Curtis et al., 1980; Brown-Borg and Edens, 1990; Nakamura et al., 1998). Similar to these authors, a significant rise in corticosterone concentration was found in the LPS treated chickens in our experiment. No significant linear relationship with the dose of the NSAID could be shown in the treated groups, although the corticosterone level in the NSAID-treated chickens seemed higher than in the positive control group. The experimental procedure that these birds experienced was not different for the NSAID-treated birds than for the positive control group.
Furthermore, the negative control group did not show levels above LOQ (2 ng/ml). This means that the experimental procedure in itself was not stressful, and did not produce a rise in corticosterone level. However, some investigators reported that simple experimental procedures (confinement, isolation and multiple blood sampling) can cause a rise in corticosterone levels in birds, such as pigeons and geese (Machin et al., 2001). The study by Cabanac and Aizawa (2000) in chickens showed an increase in body temperature and heart rate, but the birds were handled every three minutes. Therefore, it seems that the species sensitivity to stress may also play a role, and chickens might be relatively resistant to stress caused by less frequent handling. The reason for the apparent higher non-dose related corticosterone levels in the salicylate-treated chickens is not known, but evidence of adrenocortical stimulation by this drug exists (Smith and Ford-Hutchinson, 1979). This phenomenon was also seen in pigs after the intravenous administration of indomethacine, where a transient increase in cortisol was seen (Parrot and Vellucci, 1998). The higher corticosterone levels could play a role in subsequent exposures to LPS. It is hypothesised that the initial stimulation of corticosteroid secretion by endotoxin injection may protect the animal to some extent during its subsequent exposure to the toxin for there is evidence that corticosterone enhances the resistance of the fowl to *E. coli* infection (Curtis et al., 1980). This hypothesis has been supported by the findings of Koutsos and Klasing (2001). They state that an increase in glucocorticoid concentration following LPS stimulation decreases the capacity of the animal to produce pro-inflammatory cytokines.

*Ceruloplasmin*

Ceruloplasmin is an α2 glycoprotein which transports copper in the plasma and oxidises ferrous iron and various phenols and amines, including several biologically active substances such as adrenaline, noradrenaline, 5-hydroxytryptamine and histamine. This acute phase protein protects the bird against excess reactions of the immune response (Curtis and Butler, 1980). It is a positive acute phase protein, since the concentration rises after an inflammatory stimulus (Chamanza et al., 1999). Following an LPS induced acute phase reaction in chickens, a maximal rise in ceruloplasmin levels at 24 h after injection can be expected (Curtis and Butler, 1980). The rise of the ceruloplasmin plasma concentration at 24 h in the LPS injected
chickens found in our experiments is in accordance with the findings of Curtis and Butler (1980). They found a five-fold increase after 17 to 24 h, presumably due to an increased synthesis in the liver, which was partly induced by adrenal hormones. The values found by these authors are lower than the values reported here, but this can be due to the sensitivity of the method of analysis, since they used p-phenylenediamine as oxidising agent. The method of analysis in chicken plasma has been optimised by Kim and Combs (1988). Although the average value of the positive control group is larger than the NSAID-treated groups, no significant linear correlation with the NSAID dose could be shown. It seems that treatment with different doses of sodium salicylate does not have an influence on the change of concentration of ceruloplasmin after an injection with E. coli LPS.

**Thromboxane B$_2$**

The serum TxB$_2$ values were taken at 2.5 hours after the administration of the NSAID. Based on pharmacokinetic data of salicylates in chickens, the thromboxane levels should be sufficiently low at this time point. This informs us about the capacity of the NSAID to inhibit the arachidonic acid cascade. In these experiments, a significant linear relationship with the dose of the NSAID was found. The highest dose gave the largest TxB$_2$ inhibition. This value was only 3.6% of the value of the negative control animals. Interestingly, also the positive control group showed a low value for TxB$_2$. In these animals, the value for TxB$_2$ dropped to 15% of the value of the control animals. In an additional experiment, the behaviour of the TxB$_2$ concentration was studied in three groups of 5 chickens which only received a placebo treatment and an oral administration of sodium salicylate at a dose of 25 and 200 mg/kg. The group that received the 25 mg/kg SA showed practically no drop in the TxB$_2$ levels at 2.5 hours after administration of the NSAID in comparison with control animals. In the group that received the high dose (200 mg/kg), the value for TxB$_2$ fell to 13% of the value of the control animals. This means that the administration of an appropriate oral dose of sodium salicylate is effective in decreasing the TxB$_2$ values independent of the LPS injection. The low value of TxB$_2$ found in our LPS-treated chickens are in contrast to studies with IV LPS in several mammal species (Emau et al., 1985 ; Klosterhalfen et al., 1992 ; Semrad, 1993). A reason for this finding in birds is not known, but it could be a species specific response to LPS. Only one sample (at 2.5 h) was taken and it is possible that before of
after this point a rise in the TxB₂ concentration may occur. It may also be a consequence of the higher level of stress following the LPS injection, as has been proven by the high corticosterone values at this time point, which might cause an inhibition of the prostaglandin and thromboxane production (Tomchek et al., 1991). In mallard ducks, plasma levels of TxB₂ were also lowered for about 4 hours after intramuscular administration of flunixin (5 mg/kg) and ketoprofen (5 mg/kg) (Machin et al., 2001). The dosage for flunixin used in this study is higher than the dosage recommended in mammals (1.1 mg/kg). The authors chose a higher dose, since there is some evidence that flunixin does not provide analgesia at low doses in birds (Curro, 1994). Also the flunixin dosage recommended for pain relief in avian species varies from 1-10 mg/kg according to Bennet (1994). Recent findings about the pharmacokinetics of flunixin in several bird species show a very fast elimination pattern for several avian species, including ostriches and ducks (chapter 1.2.). This could partly explain the lower analgesic capacity of this NSAID in avian species. Although a very low value for serum TxB₂ was found, flunixin was not able to block the fever in the LPS fever model in broiler chickens at a dose of 1.1 mg/kg (Baert et al., unpublished results).

**Zinc**

The decrease in serum zinc is a component of the acute phase reaction. This effect is the result of an increase in metallothionein in the liver and other tissues (Klasing, 1984). This was already reported by several authors after a single LPS injection in chickens (Butler and Curtis, 1973; Johnson et al., 1993; Takahashi et al., 1995). However, the magnitude of this response varies considerably between different groups of animals and between individual animals (Butler and Curtis, 1973). Following an LPS induced acute phase reaction, a maximal decline in zinc levels at 12 h after injection can be expected (Butler and Curtis, 1973; Johnson et al., 1993; Takahashi et al., 1995). In our experiment, a decrease in zinc levels was also seen in the LPS injected chickens, but no linear relationship with increasing NSAID dose could be shown. Another important factor has to be mentioned: after repeated LPS injections in chickens, no differences in the serum zinc concentrations are found compared to control chickens (Takahashi et al., 1995). This is also seen in Japanese quail, so it seems that birds show a tolerance to LPS following repeated injections (Koutsos and Klasing, 2001). Another experiment with LPS shows that birds have a high tolerance
towards LPS. Different authors already tried to induce a Shwarzmann reaction (generalised or local) in birds, but these attempts failed (Adler and DaMassa, 1979; Katiyar et al., 1987; Mendes et al., 1994). A possible explanation for this phenomenon could be the lack of myeloperoxidase and significant oxygen radical production by avian heterophils which may account for the less severe vascular damage in birds (Mendes et al., 1994).

**Food, water and behavioural observations**

Anorexia, somnolence and reduced locomotion activity are behavioural states frequently observed in animals with acute gram-negative bacterial infections. These non-specific responses to infection are known as sickness behaviour. *E. coli* LPS injected intraperitoneally induces these symptoms in chickens (Johnson et al., 1993). Also an intravenous injection of *Salmonella* LPS in chickens induced the same sickness behaviour (Xie et al., 2000). This is also reported for an endotoxin challenge in mammals (Kozak et al., 1994). Our experiments confirm these effects of injected *E. coli* LPS via the intravenous route in chickens. However, in the experiments of Johnson et al. (1993), intraperitoneally administered indomethacin inhibited anorexia and somnolence. Our experiment failed to prove a difference in food intake between the positive control group and the NSAID-treated group. Also, no behavioural changes between the positive control group and the NSAID-treated group were seen. This is also in contrast with the LPS data collected in rats and pigs, where again indomethacin was able to completely reverse hypophagic and behavioural responses of LPS (Johnson and Vonborell, 1994; Andonova et al., 1998). This could be explained by the intrinsic activity of this particular drug, since it is known that in mice a COX2 inhibition during LPS-induced inflammation results in preserved food intake (Johnson et al., 2002). Sodium salicylate could have a more preferential action on COX1 than indomethacin at that dose. Also, the mechanism that induces the anorexia could be different in birds. Another factor, namely TNF-α, is assumed to play a role in the modulation of the food intake after an endotoxin challenge in rats (Porter et al., 2000).

In contrast with the food intake in our experiment, a significant difference was seen in the water consumption of the chickens. The NSAID-treated group drank more than the positive control group. The mechanism that regulates the water intake in animals
is still unclear. In rats, it is found that TNF-α is an important mediator for the central antidipsogenic effect of LPS. A pretreatment with intraperitoneally acetylsalicylic acid (100 mg/kg) was able to antagonise TNF-α inhibition of thirst of water deprived rats (Calapai et al., 1991). This could mean that TNF-α could be regulated by an eicosanoid dependent mechanism. But, in rats other factors such as nitric oxide (NO) and mast cells were also involved in the anti-dipsogenic effect of LPS (Nava and Caputi, 1999; Raghavendra et al., 1999). Furthermore, a dose related increase in urine flow was observed after the administration of 25-100 mg/kg of intravenous salicylate in Peking ducks. This effect is probably not related to an inhibition of prostaglandin synthetases, since other NSAIDs (indomethacin and meclophenamate) did not reproduce this effect (Gray et al., 1984). Thus, this local action of salicylate could also have caused the higher water intake in the salicylate treated birds.

CONCLUSION

Our experiments demonstrate that chickens respond to an IV injection of *E. coli* LPS by reducing food and water consumption, becoming febrile and somnolent. LPS injected chickens also show elevated plasma corticosterone and ceruloplasmin levels, while serum zinc levels decreased. These are physiological and behavioural responses also used by mammals to maintain homeostasis during infection. However, some differences such as the low value for the serum thromboxane might be considered as a species specific response to LPS. Oral sodium salicylate shows to be an effective antipyretic in chickens injected with *E. coli* LPS, when used at an appropriate dose. Other effects of the administration of this NSAID in this model are a higher consumption of water during the first hours of the febrile period.

ACKNOWLEDGEMENTS

The authors thank Hilde Lippens, Deborah Calis and Mario Schelkens for their quality technical assistance.
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General discussion
The use of non-steroidal anti-inflammatory drugs (NSAIDs) in veterinary medicine has increased considerably. Since the early 70's, several NSAIDs were routinely used in the treatment of febrile states and inflammation disorders of several animal species, including cats, dogs, horses, swine and cattle (Yeary and Swanson, 1973; Mathur et al., 1974; Barber et al., 1974; Yeary and Brant, 1975; Fagot, 1975; Gingerich et al., 1975; Eyre et al., 1976). Later, other indications such as endotoxic shock and colic in the horse and the control of pain, associated with trauma or operation were found to be positively influenced by NSAIDs (Boothe, 2001). Salicylates may be a useful class of drugs for veterinary medicine since these drugs are cheap and can easily be used in food and drinking water medication. Several authors have proposed indications for the use of NSAIDS in bird medicine, but a lot of the information available is contradictory or empirical. For many of these indications, the benefits of an NSAID treatment have not been clearly proven or relevant experiments led to negative results.

In broiler production systems, NSAIDs were proposed to be beneficial in digestive parasitic infections (Hornok et al., 1999; Allen, 2000; Cristofol et al., 2000; Vermeulen, 2002). The mechanism of action of this effect on coccidiosis was hypothesised to be a PG dependent mechanism, since an increased prostaglandin biosynthesis might be suspected in coccidial infections, mediated by a nitric oxide (NO) induced increased activity of COX2 (Vermeulen, 2002). The mechanism of action in the pathogenesis of Cryptosporidium baileyi was also linked to PGs by the authors, since NSAIDs can restore the IL-2 levels during protozoan infections in mammals. Prostaglandins are often associated with parasite-induced immunosuppression via inhibition of IL-2 secretion (Hornok et al., 1999). Sudden death syndrome in turkeys and heavy breed chickens and broiler ascites have been suggested as indications for salicylate treatment. The thromboxane and prostaglandin inhibitory effect of salicylate would prevent blood clotting in turkeys and broilers. However, no positive effects on these syndromes were found (Proudfoot and Hulan, 1983; Boulianne and Hunter, 1990; Balog et al., 2000). Several authors have found a beneficial effect of salicylates on the growth and egg production of chickens during heat stress, claiming a deleterious effect of PG during high environmental temperatures (Oluyemi and Adebajo, 1979; Oliver and Birrenkott, 1981; Abd-Ellah et al., 1997). However, other researchers were not able to confirm these results (Mathur et al., 1974; Stillborn et al., 1988). Other investigators studied the effects of salicylates in feed of laying hens. It was hypothesised that prostaglandins may be
responsible for the premature expulsion of some soft-shelled (SS) or shell-less (SL) eggs. A reduction in the production of SL eggs in favour of the SS eggs was found but no changes were found in other parameters (egg production, time of oviposition, behaviour of birds, fertility and hatchability of embryos) (Gilbert et al., 1982; Balog and Hester, 1991; McDaniel et al., 1993a; McDaniel et al., 1993b). Other indications based on the anti-inflammatory and analgesic capacities of NSAIDs seem more logical. In cases of locomotion disturbances of degenerative or infectious nature or trauma NSAIDs may be very useful (Hocking, 1988; Jouglar and Benard, 1992; Danbury et al. 1997; Mc Geown et al, 1999). It is clear that the search for a valid indication in poultry industry is not evident. Economical factors play a major role in treatment of diseases and the choice of drugs in these species.

Information about pharmacokinetics of NSAIDs in birds is very scarce. The pharmacokinetics of ibuprofen in broiler chickens was investigated by Roder et al. (1996) and Vermeulen and Remon (2001). The indomethacin pharmacokinetics after oral and intravenous administration in poultry was reported by Cristòfol et al. (2000). In this thesis, the intravenous pharmacokinetics of three common veterinary NSAIDs (sodium salicylate, flunixin and meloxicam) were studied in five food producing bird species (chickens, turkeys, ostriches, ducks and pigeons). The intravenous route of administration was chosen, because the absorption process is bypassed and pharmacokinetic parameters can be calculated accurately. A large interindividual variability in plasma concentrations and pharmacokinetic parameters of ibuprofen and indomethacin is found in broiler chickens, even after intravenous administration (Cristòfol et al., 2000; Vermeulen and Remon, 2001). The experiments described in chapter 1.2. with the three NSAIDs showed little variability among individuals of the different bird species. It is known from pharmacokinetic experiments with NSAIDs in mammals that large species differences can occur (Boothe, 1989). This was also seen in our experiments between these bird species (chapter 1.2.). Flunixin has a very short half-life in most investigated bird species, except for chickens. In these birds, the average half-life was more than 5 h. The half-life of meloxicam was generally longer than for flunixin, but still very fast for ostriches. For salicylate, the half-life in pigeons was very long compared to the other bird species. Again the ostrich shows the shortest half-life. An important factor in this issue can be the difference in protein binding capacity of drugs in the studied bird species. The volume of distribution for flunixin is
low for all of the bird species. This could mean that the protein binding for flunixin is high for all the bird species. The volume of distribution for meloxicam and salicylate in the different species, however, shows a larger variation. The volume of distribution for meloxicam in ostriches is at least three times larger than the other species. The volume of distribution for salicylic acid is high for all the bird species compared to some mammal species except for the ostrich. These findings can be explained by a different protein binding for that drug in that specific bird species. Based on the protein-binding study in chapter 1.2., some of these hypotheses can be substantiated with our results. The pigeon has a very low plasma protein binding for salicylic acid. As a result, the concentration of free drug in the plasma is high and can be readily distributed into the tissues. This is reflected by the high volume of distribution for salicylic acid in pigeons. However, other bird species also have a high volume of distribution and do not show such a low protein binding for salicylic acid. This means that other factors must play a role in this phenomenon. A different tissue binding capacity can be a reason for this difference in volume of distribution.

Further pharmacokinetic research can also be focused on another manner of administration since oral administration of drugs via feed or drinking water to large flocks of birds is preferred. Moreover, the absorption process of drugs in birds is relatively unknown and the anatomical differences in the upper part of the gastrointestinal system (crop, glandular stomach and ventriculus) might have an influence on this process (Roder et al., 1996; Vermeulen and Remon, 2001). Also the presence of food may be an important factor. This has been demonstrated for several NSAIDs including phenylbutazone, fenoprofen and flunixin in horses, where presence of hay can play a role in the variable absorption kinetics (Tobin et al., 1986; Delbeke et al., 1995). Other important issues are the drug disposition after multiple or continuous administration and the possible adverse reactions associated with the drugs. In mammal species, it is known that NSAIDs have a number of possible adverse effects that are induced by their mechanism of action. The inhibition of constitutive cyclooxygenase (COX1) is responsible for gastro-intestinal damage with formation of ulcer, for hematopoietic disturbances of platelet activity, for nephropathy (mostly in combination with other factors, such as hypovolemia, other renal diseases or after nephrotoxic drug administration). Other miscellaneous effects are hepatotoxicity, diarrhoea and influence on the central nervous system (Boothe, 2001). Some NSAIDs have shown species specific adverse reactions. Ibuprofen is a relatively safe drug in
humans, but dogs, especially German Shepherds, seem to be highly susceptible to the gastro-intestinal side effects of ibuprofen (Poortinga and Hungerford, 1998). In humans, hypersensitivity to acetylsalicylic acid is known as the Reye's syndrome. The symptoms consist of altered consciousness, convulsions, hypoglycaemia and hepatomegaly associated with fatty infiltration of the liver. It has been known to occur at therapeutic doses in children with mild viral infections (Clark et al., 2001). In broiler chickens, the intravenous administration of ibuprofen at a dosage of more than 50 mg/kg seems to result in acute toxicity and death (Roder et al., 1996). The single intravenous administration of sodium salicylate (25 mg/kg), meloxicam (0.5 mg/kg) and flunixin (1.1 mg/kg) in these different bird species did not result in clinical adverse effects. Only the rapid administration of flunixin in ostriches resulted in a transient depression of the birds. A similar reaction can be seen when the same drug is rapidly injected intravenously in horses (Dr. J. Nackaerts, 1998, personal communication). Sodium salicylate possesses similar antipyretic and anti-inflammatory properties as acetylsalicylic acid, but lacks the inhibitory effect on the activity of isolated cyclo-oxygenases (in the platelets). This means that for sodium salicylate, no side effects on the hematopoietic system can be seen and no prolonged bleeding time can be expected. It was also shown that sodium salicylate, although it might act as a mild irritant on gastric mucosa, lacks ulcerogenicity in experimental animals (Amann and Peskar, 2002). These possible adverse effects obviously need further attention in the different bird species. Based on the pharmacokinetics of salicylates in birds, the knowledge on adverse effects of sodium salicylate in mammals together with its economical advantages, it seems that this NSAID might have a high potential for use in poultry industry. Also, the withdrawal time for substances which have a maximum residue limit (MRL) should be calculated. Salicylates are listed in annex II and do not have an MRL. This means that these drugs can be readily used in food producing species, without establishing a withdrawal time. However, at the moment, no registered formulations are available in Belgium. Flunixin and meloxicam both have an MRL for food producing species and may be used according to the cascade protocol, if an appropriate withdrawal time is used. Both drugs are expensive and specific formulations for administration in feed or drinking water are not available. Sodium salicylate is readily soluble in drinking water and can easily be administered. Acetylsalicylic acid is not water soluble and not stable and should be administered in feed unless it is combined with an amino acid (e.g.
lysine or arginine). This type of formulation was also used to improve the water solubility of ibuprofen before administration in broiler chickens (Vermeulen, 2002).

Another factor that can be a possible reason for the species difference in pharmacokinetics may be the variations in biotransformation of the drug. In this thesis, the excretion pattern of amino acid conjugation metabolites of salicylic acid was investigated in broiler chickens and homing pigeons. A marked species difference was found. Chickens conjugated salicylic acid with ornithine as amino acid and pigeons used glycine. Another interesting finding was the fact that the glycine conjugate (salicyluric acid) could not be detected in the plasma of the pigeons, but large amounts were recovered in the faeces. Analysis of tissues showed that the main site of glycine conjugation was located in the pigeon kidney. Thus, it seems that formation of the conjugate occurred immediately before excretion in the urine of the pigeon, without or with minimal reabsorption into the blood stream. In chickens, the ornithine conjugate is detected in liver as well as in kidney and formation seems not to be limited to one site.

In the second part of the thesis, the efficacy of an oral salicylate treatment in an inflammation in chickens has been investigated. Due to the paucity of information about inflammation in birds, there is an obvious need to develop useful inflammation models to investigate the pharmacodynamics of NSAIDs in birds. Two different inflammation models were developed to obtain information about the pharmacodynamics of salicylate in chickens. The first model was the subcutaneous insertion of a foreign object (sponge), treated with carrageenan. This model was based on the work of Higgins and Lees (1984) in horses and of Chansoriya et al. (1994) in chickens. The first research group further developed the model in horses and expanded it to another animal species (calves) (Landoni et al., 1995). The main advantage of this method was the collection of inflammatory exudate. In this exudate, a number of pharmacokinetic and pharmacodynamic parameters can be assessed. In our experiments, the pharmacokinetics of oral salicylate in chickens were studied via the plasma concentrations and also the concentration in the exudate was measured. It was clear that the concentration of salicylic acid in the exudate was higher than the concentration in the plasma and remained high during a longer period of time. Nevertheless, it must be stated that the higher levels of salicylic acid in the exudate
may be the result of an accumulation of salicylic acid. To study this effect further, another type of foreign object could be used. A hollow ball, where exudate can be sampled on several occasions might be a better solution. This was already done successfully by Higgins et al. (1987). They could confirm the longer half-life of different NSAIDs in the inflammatory exudate in their horse and calf inflammation model. Obviously, in chickens, a more appropriate size of the ball should be chosen. In our experiments, only a difference in PGE$_2$ concentration between SA treated and non-treated chickens was found at the first time point (4 h) of the exudate sampling. This is in contrast with studies in mammals. It seems that the administration of SA in chickens can play a role in preventing PG formation at the site of inflammation, but it could also mean that prostaglandins only play a role in the acute phase of the inflammation and that the values rapidly fall back to basic levels. The role of the eicosanoid pathway in the inflammatory response in birds needs further attention. Based on our experiments, it seems that bradykinin is not a major mediator. Practically no swelling was seen after intradermal injection in the chicken skin. Other authors suggest that serotonin and histamine play an important role (Ito et al., 1989). The other inflammation parameters, volume of exudate and leukocyte numbers, were not affected by SA treatment. These parameters, however, are not very specific. The volume of exudate being a measure of increased vascular permeability and leukocyte numbers of the cellular migration. Although it is known that sodium salicylate has an inhibitory effect on leukocyte migration in mammalian neutrophil models in vitro (Pierce et al., 1996), no difference could be shown in our in vivo chicken inflammation model. The oral dosage of 50 mg/kg could be insufficient for obtaining an effect on leukocyte migration. It would be interesting to study the in vitro effects of NSAIDs on the inflammatory properties of the avian heterophil. In conclusion, this inflammation model offers possibilities for further research, but evidence for the beneficial pharmacodynamic properties of NSAIDs in this model are moderate.

A second model was used to investigate the pharmacodynamics of sodium salicylate in chickens. An *E. coli* lipopolysaccharide (O127:B8) which has reproducible pyretic properties in broiler chickens was selected from the literature. The administration of different oral dosages of sodium salicylate to different treatment groups showed a dose dependent attenuation of the fever, induced by the LPS. These results clearly show the antipyretic properties of sodium salicylate in chickens. Several other parameters that are known to change during an LPS-induced acute phase response in
chickens were monitored, but the effects of the NSAID on these parameters were not dose dependent, except for serum thromboxane B$_2$, which is increasingly lowered with increasing dose. This means that the mediators resulting from the arachidonic acid cascade reaction are reduced after salicylate treatment in birds. But the decrease in serum thromboxane B$_2$ after an LPS injection (the positive control group) seems to be a peculiar feature in chickens in comparison to mammalian species. This could also mean that the mediation of inflammatory disorders in birds is somewhat different than in mammals. The concept of the inflammatory modulators in mammals was described by Van Miert (2002) as a very complicated and dynamically regulated syndrome, in which many processes are induced and turned on or off. This modulation could be slightly different in birds, but still have the same result, namely resolution of inflammation. The role of corticosterone in this modulation process is not clear, but plasma concentrations after sodium salicylate treatment seemed higher, independent of SA dosage. Salicylates can induce a rise in cortisone in mammals. It is clear that the mediation of the acute phase reaction in birds is not well defined. Further research on this topic could reveal interesting properties of bird inflammation. A special feature of the sodium salicylate administration after an LPS injection seems to be the difference in water intake. The water intake of the salicylate treated birds was intermediate between the positive and the negative control group. Reasons for this difference remain unclear, but a direct diuretic action of salicylic acid on the avian kidney cannot be excluded. In contrast, the food intake was not altered, although other authors found clear differences in other animal species.

These pharmacodynamic studies show that NSAIDs have a potential for use in bird medicine. However, these results are obtained in simple inflammation models. In bird medicine, a lot of complicating factors exist and the influence of NSAIDs on the course of the diseases, sometimes complicated by different infectious and environmental factors, remains to be investigated.

The general conclusions of this research in the field of the pharmacokinetics and pharmacodynamics of NSAIDs in bird medicine are manifold: the investigated NSAIDs (sodium salicylate, flunixin and meloxicam) generally have a short elimination half-life, but large species differences exist. The pharmacokinetics of other more practical routes of administration need further research. The differences in biotransformation may be a reason for the differences in pharmacokinetic parameters.
GENERAL DISCUSSION

Salicylates have a significant antipyretic effect in chickens, but the mediators of inflammation in birds and the influence of NSAIDs on existing diseases and pain need further research.
REFERENCES


The aims of this thesis were to investigate the pharmacokinetics and pharmacodynamics of non-steroidal anti-inflammatory drugs in birds. For decades, the inflammatory process and the properties of the NSAIDs have been extensively investigated in humans and other species. However, current knowledge on inflammation and anti-inflammatory drugs in birds is limited. Inflammation is a complex mechanism designed to protect the organism against different stimuli. NSAIDs act as inhibitors of the formation of certain mediators of inflammation, originating from the arachidonic acid cascade. In the general introduction, the literature is reviewed about the mechanism of action, the adverse reactions and the pharmacokinetics of non-steroidal anti-inflammatory drugs, and the possible indications for use of anti-inflammatory drugs in bird medicine. Due to the lack of information on NSAIDs in birds, most of the information about the mechanism of action and the adverse reactions are described based on the knowledge obtained in mammal species. In chapter 1.1., an overview about the main pharmacokinetic parameters and some bird specific anatomy and physiology is given. In chapter 2.1., the literature about the different aspects of inflammation in birds is reviewed: increased vascular permeability, leukocyte changes, acute phase proteins and inflammation mediators. On several occasions, a comparison with inflammation in mammals is made. Especially the acute phase of inflammation is described.

Information on the pharmacokinetics of anti-inflammatory drugs in birds is scarce. Choice of drug and choice of dosage is usually empirical, since studies of anti-inflammatory drugs are lacking. In chapter 1.2., the first experiments are described, three commonly used veterinary non-steroidal anti-inflammatory drugs (NSAIDs) were administered intravenously to five different bird species. Sodium salicylate, flunixin and meloxicam were selected as anti-inflammatory drugs.
These NSAIDs were administered intravenously to chickens (Gallus gallus), ostriches (Struthio camelus), ducks (Cairina moshata), turkeys (Meleagris gallopavo) and pigeons (Columba livia). Plasma concentrations of the drugs were determined by validated high-performance liquid chromatography methods and pharmacokinetic parameters were calculated with compartment models. Also the plasma protein binding capacity of the different bird species for flunixin, meloxicam and salicylic acid at three different concentrations was studied. Most bird species exhibited a rapid elimination of these drugs. Ostriches had the fastest elimination rate for all three NSAIDs, but there were some interesting species differences. For salicylic acid, the half-life in pigeons was at least three to five times longer than the other bird species. Chickens had a half-life that was approximately ten times as long as the other bird species for flunixin and the half-life of meloxicam in chickens and pigeons was three times as long as for the other bird species. The plasma protein binding capacity for flunixin and meloxicam was moderate and showed a similar pattern for the different bird species. For salicylic acid more variation was seen between the species and pigeons exhibited a very low plasma protein binding capacity for salicylic acid.

Several reasons for this difference in pharmacokinetic parameters are possible. Metabolism of these drugs can be species dependent. Therefore, the excretion pattern of sodium salicylate and its metabolites in chickens and pigeons was studied. This is described in chapter 1.3. An intravenous injection was made in these birds and the combined urinary and faecal droppings were collected at different time intervals. A marked difference was seen in the amino acid conjugation pattern of salicylic acid. Chickens used ornithine for conjugation and pigeons used glycine. Furthermore, the excretion of the glycine conjugate in pigeons was prolonged and in contrast to the ornithine conjugate in chickens, was not seen in the plasma. Based on preliminary information obtained in liver and kidney tissues of these species, it seems that the amino acid conjugation of salicylic acid with glycine in pigeons occurs only in the kidney and the amino acid conjugation of salicylic acid with ornithine in chickens occurs both in liver and in kidney.

The pharmacodynamics of the NSAIDs in birds were studied in two inflammation models in chickens. In both models, sodium salicylate was used as NSAID, since this
drug exhibited favourable pharmacokinetics in chickens and can be economically and practically interesting for use in poultry medicine.

In chapter 2.2., an experiment where an acute inflammatory reaction was generated by implanting a carrageenan impregnated sponge strip in the subcutaneous tissue of broiler chickens is described. Half of the chickens received 50 mg/kg BW sodium salicylate orally and half received a placebo treatment. Blood and exudate samples were taken from these chickens at predetermined times. The pharmacokinetics of sodium salicylate were investigated in blood and exudate. Other parameters that were investigated were the volume of the exudate, the number of leukocytes in the exudate and the prostaglandin E\textsubscript{2} concentration in the exudate. Also bradykinin was injected intradermally and the effects of sodium salicylate on bradykinin induced oedema were studied. Maximum salicylic acid plasma concentrations occurred within the hour after administration and maximum exudate levels were seen at the first exudate sampling point (4 h). Salicylic acid exudate concentrations exceeded plasma concentrations at the same time points and the exudate half-life was longer than the plasma half-life. No differences were found in the volume of the exudate and the leukocyte numbers between the treated and the untreated group. Sodium salicylate reduced the PGE\textsubscript{2} concentration in the inflammatory exudate at the 4 hour time point, but at the later time points, no significant differences were found. The intradermally injected bradykinin did not produce significant effects in the chicken skin. Based on these findings, sodium salicylate may be used to suppress the inflammatory cascade in chickens, but further research is needed to characterise the inflammatory response in this chemical inflammation model and the actions of this non-steroidal anti-inflammatory drug in chickens.

In chapter 2.3., the last set of experiments are described. An acute phase reaction in broiler chickens was provoked through the intravenous injection of \textit{Escherichia coli} lipopolysaccharide. Two experiments were set up with sodium salicylate to study the effects of this NSAID upon the LPS acute phase reaction. In the first experiment, different oral doses of sodium salicylate were given and the effect on body temperature was measured. Other inflammation indices, such as plasma corticosterone and ceruloplasmin levels, serum thromboxane B\textsubscript{2} and zinc levels were also monitored during the experiment. In a separate experiment, food and water consumption and
other behavioural parameters were studied. In the first experiment, a dose dependent attenuation of the fever response of the chickens in the salicylate treated groups was observed. Plasma corticosterone and ceruloplasmin levels rose and zinc and thromboxane B2 levels declined after an LPS injection. Except for thromboxane B2, no dose dependent differences after treatment with sodium salicylate were seen in these parameters. In the second experiment, the water intake, but not the food intake, was significantly higher in the salicylate treated group. No behavioural differences were seen between the positive control and the salicylate treated group. These data confirm that sodium salicylate is an effective antipyretic agent after injection of LPS in chickens, if used at an appropriate dosage.

The general conclusions of this research after the pharmacokinetics and pharmacodynamics of NSAIDs in bird medicine are manifold: the investigated NSAIDs generally have a rapid half-life, but large species differences exist. The pharmacokinetics of other more practical administration routes need further research. The differences in metabolism may be a reason for the differences in pharmacokinetic parameters between the bird species. Salicylates have a clear antipyretic effect in chickens, but the mediation of avian inflammation and the influence of NSAIDs on disease and pain need further research.
De doelstelling van deze thesis was het onderzoek naar de farmacokinetiek en farmacodynamiek van niet-steroidale anti-inflammatoire geneesmiddelen (NSAID's) bij vogels. Het ontstekingsproces en de eigenschappen van NSAIDs werden al gedurende tienallen jaren onderzocht bij mensen en andere zoogdieren. De huidige kennis over ontsteking en ontstekingswerende middelen bij vogels is echter beperkt. Ontsteking is een complex mechanisme dat het organisme beschermt tegen verschillende stimuli. NSAIDs werken als inhibitoren op de vorming van bepaalde onstekingsmediatoren die ontstaan uit de arachidonzuur cascade reactie. In de algemene inleiding wordt een overzicht gegeven van het werkkingsmechanisme, de mogelijke indicaties voor gebruik van anti-inflammatoire geneesmiddelen bij vogels en de neveneffecten van NSAIDs. De meeste gegevens die beschikbaar zijn in de literatuur over het algemeen werkkingsmechanisme en de neveneffecten zijn gebaseerd op onderzoek bij zoogdieren, aangezien er weinig onderzoek bij vogels hierover is gepubliceerd. In hoofdstuk 1.1. wordt een overzicht gegeven van de belangrijkste farmacokinetische parameters en van enkele anatomische en fysiologische aspecten specifiek voor vogels. In hoofdstuk 2.1. wordt een overzicht gegeven van de verschillende aspecten van ontsteking bij vogels: verhoogde vasculaire permeabiliteit, veranderingen in witte bloedcellen beeld, acute fase eiwitten en mediatoren van ontsteking. Op verschillende vlakken wordt een vergelijking met de situatie bij zoogdieren gemaakt. Vooral de acute fase van ontsteking wordt beschreven.

Gegevens over de farmacokinetiek van niet-steroidale anti-inflammatoire geneesmiddelen zijn zeldzaam. De keuze van het geneesmiddel en de keuze van de dosis is gewoonlijk empirisch aangezien specifieke studies over NSAIDs bij vogels ontbreken. In hoofdstuk 1.2. worden de eerste experimenten beschreven. Drie NSAIDs die frequent gebruikt worden in de diergeneeskunde, werden intraveneus toegediend aan vijf verschillende vogelsoorten. Natrium salicylaat, flunixine en meloxicam werden gekozen als niet-steroidale anti-inflammatoire geneesmiddelen.
Deze NSAIDs werden intraveneus toegediend aan kippen (*Gallus gallus*), struisvogels (*Struthio camelus*), eenden (*Cairina moshata*), kalkoenen (*Meleagris gallopavo*) en duiven (*Columba livia*). De plasma concentraties van deze geneesmiddelen werden nagegaan met gevalideerde hoge druk vloeistof chromatografie methodes en farmacokinetische parameters werden berekend met compartimentele methoden. Ook de plasma eiwit binding van flunixine, meloxicam en salicylzuur werd bepaald op drie verschillende concentraties voor de vijf vogelsoorten. De meeste vogels vertoonden een snelle eliminatie van deze geneesmiddelen. Struisvogels hadden de snelste eliminatiesnelheid voor de drie NSAIDS, maar er waren interessante species verschillen. De halfwaardetijd van salicylzuur bij duiven was op zijn minst drie tot vijf keer langer dan de andere vogelsoorten. De halfwaardetijd van flunixine was ongeveer tien keer langer bij kippen dan bij de andere vogelsoorten en de halfwaardetijd van meloxicam bij kippen en duiven was ongeveer drie keer zo lang als voor de andere vogelsoorten. De plasma eiwitbinding was middelmatig voor flunixine en meloxicam en vertoonde een gelijkaardig patroon voor de verschillende vogelsoorten. Voor salicylzuur werd er meer variatie gezien tussen de soorten en duiven vertoonden een zeer lage plasma eiwitbinding.

De farmacodynamiek van NSAIDs bij vogels werd bestudeerd in twee inflammatoire modellen bij kippen. In beide modellen werd natrium salicylaat gebruikt als NSAID, aangezien deze stof gunstige farmacokinetische eigenschappen vertoonde bij kippen en deze stof economisch en praktisch interessant kan zijn voor gebruik in de geneeskunde van vogels.

In hoofdstuk 2.2. wordt een experiment beschreven waarbij een acute ontsteking opgewekt werd door de implantatie van een met carrageenan geïmpregneerde spons in het subcutane weefsel van mestkippen. De helft van de kippen werd oraal behandeld met 50 mg/kg natrium salicylaat en de andere helft kreeg een placebo toegediend. Bloed- en exudaatstalen werden op vooraf vastgelegde tijdstippen genomen. Andere parameters die werden onderzocht, waren het exudaat volume, het aantal leucocyten in het exudaat en de prostaglandine E2 concentratie in het exudaat. Ook werd bradykinine intradermaal geïnjecteerd en werden de effecten van salicylaat op bradykinine geïnduceerd oedeem onderzocht. De maximale plasmaspiegels van salicylzuur werden binnen het uur na toediening waargenomen en de maximale spiegels in het exudaat werden gezien na de eerste exudaat staalname (4 h). De concentraties van salicylzuur in het exudaat waren hoger dan de plasmaspiegels op dezelfde tijdstippen en de halfwaardetijd was langer in het exudaat dan in het plasma. Er werden geen verschillen gevonden in het exudaat volume en de aantallen witte bloedcellen tussen de behandelde en de onbehandelde groep. Natrium salicylaat verminderde de concentratie aan PGE2 in het ontstekings exudaat op het 4 h tijdstip, maar op de latere tijdstippen werden geen significante verschillen gevonden. Het intradermaal geïnjecteerde bradykinine gaf geen duidelijke effecten in de dermis van kippen. Gebaseerd op deze bevindingen kan natrium salicylaat gebruikt worden om de ontstekingscascade te onderdrukken bij kippen, maar verder onderzoek is nodig om de ontstekingsreactie die geïnduceerd wordt door dit chemisch ontstekingsmodel en de eigenschappen van dit anti-inflammatorisch geneesmiddel bij kippen te karakteriseren.

In hoofdstuk 2.3. worden de laatste experimenten beschreven. Er werd een acute fase reactie uitgelokt bij mestkippen met de intraveneuze injectie van Escherichia coli lipopolysaccharide. Twee experimenten werden uitgevoerd met natrium salicylaat om de effecten van dit NSAID op een LPS acute fase reactie te bestuderen. In het eerste
experiment werden verschillende orale dosissen van natrium salicylaat gegeven en het effect op de lichaamstemperatuur werd gemeten. Ook andere ontstekingsindicatoren, zoals plasma corticosterone en ceruloplasmine concentraties, serum thromboxane B₂ en zink concentraties werden gevolgd tijdens het experiment. In een aparte proef werden voeder- en wateropname en andere gedragsparameters onderzocht. In het eerste experiment werd een dosisgebonden vermindering van de koortsreactie gezien bij de kippen in de met salicylaat behandelde groepen. De plasma corticosterone en ceruloplasmine gehalte stegen en de zink en thromboxane B₂ gehalte daalden na een LPS injectie. Behalve voor thromboxane B₂ werden geen dosisgebonden verschillen gezien na behandeling met natrium salicylaat. In het tweede experiment was de wateropname, in tegenstelling tot de voederopname, significant hoger in de met salicylaat behandelde groep. Geen verschillen in gedragsparameters werden geconstateerd. Deze gegevens bevestigen dat natrium salicylaat een goed werkend koortsverend middel is na de injectie van LPS bij kippen, indien het gebruikt wordt aan een voldoende hoge dosis.

De algemene conclusies van dit onderzoek naar de farmacokinetiek en de farmacodynamiek van NSAIDs in de vogelgeneeskunde zijn veelvoudig: de onderzochte NSAIDs hebben over het algemeen een snelle halfwaardetijd, maar er bestaan grote diersoortverschillen. De farmacokinetiek van eenvoudiger manieren van toediening zou verder onderzocht moeten worden. Het verschil in biotransformatie kan een oorzaak zijn van het verschil in farmacokinetische parameters tussen de vogelsoorten. Salicylaten hebben een koortsverend effect bij kippen, maar de mediatie van ontsteking bij vogels en de invloed van NSAIDs op ziekte en pijn zou verder onderzocht moeten worden.
DANKWOORD

Dit proefschrift bevat het werk dat over een tijdspanne van meer dan zes jaar opgebouwd is. Het is dan ook niet verwonderlijk dat een groot aantal mensen rechtstreeks of onrechtstreeks geholpen hebben om dit tot een goed einde te brengen.

Mijn grote waardering gaat uit naar mijn promotor, Prof. Dr. Patrick De Backer. Hij heeft zijn interesse in de farmacologie aan mij doorgegeven en mij de mogelijkheden gegeven om dit uit te bouwen in een diersoort die ons beiden nauw aan het hart ligt. Dank zij zijn aanmoedigingen en waardevolle suggesties is dit proefschrift tot een goed einde gekomen. Daarnaast heeft hij mij ook alle kansen gegeven om mij in te werken in de kinetiek en residu problematiek van diergeneesmiddelen en de diergeneeskundige toxicologie. Patrick, bedankt voor al het vertrouwen dat je in mij stelt.

Peter en Siska, de tijd die we hier samen hebben doorgebracht was fantastisch. Jullie enthousiasme en vriendschap hebben me door enkele moeilijke momenten geloodst en maakten dat de rest van de tijd gewoonweg een plezier was. De voorbije zeven jaar waren zonder twijfel 'zeven vette jaren'!

Kris, van jou zou ik een tweetal dingen willen eruit halen. De beginselen en het onderhoud van het HPLC toestel heb ik grotendeels van jou geleerd. Je stond altijd klaar om me te helpen met een drukschommeling of een verstopte leiding. Verder is jouw onuitputtelijke energie een stimulans geweest om gedurende al die jaren mijn conditie op een aanvaardbaar peil te houden om samen te kunnen lopen, fietsen of pompen. Ik zou je hiervoor minstens 'veertig' keer willen bedanken!

Maggy, de liefde voor de toxicologie heb ik zeker van jou overgenomen. De manier waarop jij hier zowel de routine analyses voor kinetiek als toxicologie aanpakt is ongelofelijk en kan als voorbeeld dienen voor iedereen.

Een bijzonder plaats in het dankwoord is weggelegd voor Hilde. Dankzij jouw hulp zijn de experimenten altijd vlot verlopen. Zelfs de staalnames ’s avonds laat schrokken jou niet af! Kleine Fernand, je geniet al eventjes van een welverdiend pensioen, maar toch wil ik je bedanken voor het in mekaar zetten van de kippenstal. Hij doet nog altijd dienst.
Verder zou ik Dr. Johan Nackaerts willen bedanken. Door het kosteloos beschikbaar maken van de struisvogel als proefdier heb je zonder het te weten een zeer grote bijdrage geleverd aan het tot stand komen van dit proefschrift. Ook mijn thesisstudenten Frederik Oosterlinck, Raf Somers en enkele andere vrijwilligers waren een grote hulp tijdens die experimenten.

Marc, An en Mario, jullie hebben met jullie kennis over massaspectrometrie en ELISA dit werk een diepere dimensie gegeven. Bedankt!

Mijn dank gaat ook uit naar Prof. Dr. Serge Van Calenbergh, Ulrik Hillaert en Prof. Dr. Luc Duchateau die mij in de eindsprint van het proefschrift zeer goed geholpen hebben.

Natuurlijk wil ik ook de leden van de begeleidingscommissie danken: Prof. Dr. Romain Lefebvre, Prof. Dr. Jean-Paul Remon, Prof. Dr. Rik Ducatelle en Prof. Dr. Evelyne Meyer. Jullie waardevolle opmerkingen maakten het werk completer.

Em. Prof. Dr. Adelbert Van Miert en Prof. Dr. Christian Burvenich dank ik voor het kritisch nalezen van stukken van het proefschrift en hun werk als lid van de examencommissie. Ook de andere leden van de examencommissie, Prof. Dr. Aart De Kruijf, Prof. Dr. Hubert De Brabander, Prof. Dr. Luc Duchateau en Dr. Kathleen Hermans, zou ik hiervoor willen bedanken.

Mijn dank gaat uiteraard ook uit naar de andere collega's van de vakgroep, Siegrid, Leo, Ann, Edith en Pascal van de kinetiek groep en Frans, Noël, Trui, Dominique, Wim, Koen en Petra van de doping groep. Aan de jonge doctorandi Leo, Wim en Koen wens ik veel succes en doorzettingsvermogen in het behalen van hun doctoraat. Het dopingcontroelaboratorium wens ik veel succes op hun nieuwe locatie en hoop dat de contacten en eventuele samenwerking niet zullen afgebroken worden.

De collega's assistenten Gunther, Ann, Sofie, Lieven, Dominic, Bart en vele anderen bedank ik ook voor hun interesse en steun. Jullie hebben ons veel vriendschap gegeven.

Verder wil ik mijn vrienden dierenartsen, vrienden van de conditietraining en tennisclub en buren bedanken omdat zij helpen om het leven gezellig te maken.

Tot slot wil ik mijn familie en schoonfamilie bedanken. In het bijzonder mijn ouders, die mij altijd gesteund en aangemoedigd hebben. Mijn papa zei altijd: 'je mag studeren tot je dertig bent'. Hij had waarschijnlijk nooit verwacht dat het zelfs iets langer zou worden. Mijn mama stond en staat nog altijd klaar voor ons als we haar nodig hebben. Bedankt!
Mijn broers Tom en Wim wil ik ook bedanken, in het bijzonder Wim, die een grote hulp was tijdens de experimenten met de eenden. Mijn grootouders Noël en Alma verdienen ook een speciaal plaatsje. Het was ongetwijfeld tijdens een van onze vakanties daar dat ik mijn interesse in de wetenschap heb ontwikkeld. Peter Noël, jouw nieuwsgierigheid in de anatomie en fysiologie van de dieren en jouw drang naar kennis hebben mij toen reeds in deze richting geleid. Maar de belangrijkste personen in mijn leven zijn natuurlijk mijn vrouwtje Bene, en mijn twee zoontjes Lars en Paco. Hoewel ik zeer graag naar mijn werk kom, ik ben altijd nog gelukkiger dat ik naar huis, naar jullie, kan gaan.

Kus


Op 9 januari 2001 behaalde Kris Baert het getuigschrift van de doctoraatsopleiding in de diergeneeskundige wetenschappen. Hij is auteur of mede-auteur van 23 publicaties in internationale en nationale tijdschriften en nam actief deel aan verschillende internationale congressen.
List of papers:


